DESIGNING AND CHARACTERIZING THE PLANT-BASED CHITOSAN-MODIFIED POLY (LACTIC-CO-GLYCOLIC ACID) NANOPARTICLES FOR *SALMONELLA* AND *E. COLI*-INDUCED GASTROENTERITIS

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ABSTRACT

A global mortality rate of over 1.6 million deaths was reported in 2017 due to gastroenteritis. The highest mortality rate is reported annually in sub-Saharan Africa and South Asia. In Namibia, the national prevalence of gastroenteritis was 17% in 2014 and to date, gastroenteritis is responsible for 5% of all deaths in children under the age of 5. In the Ohangwena region, 23.8% of children were reported to have gastroenteritis from a total sample size of 530 children in 2020. *Salmonella* species and *Escherichia coli* were responsible for most bacterial gastroenteritis cases reported in Windhoek in 2018. Some of the key challenges in the management of gastroenteritis are: 1) Antibiotics and antidiarrheal agents used in the treatment of gastroenteritis have side effects in children and immunocompromised patients. 2) The high treatment doses required for the effective treatment of gastroenteritis are harmful to the beneficial microbiota within the digestive system. 3) *Salmonella* species and *E. coli* which cause gastroenteritis, have shown over 60% resistance to the available antibiotics. 4) Many orally administered antibiotics and antidiarrheal agents have reduced bioavailability at the site of infection, within the alimentary canal. Extracts derived from medicinal plants are used in ethnomedicine in African countries including Namibia as alternative treatments for *Salmonella* and *E. coli*-induced gastroenteritis. However, the lack of information on the presence and quantity of bioactive compounds present, their safety, and efficacy. Moreover, plant-based treatments are faced with numerous challenges within the GI tract which reduce their bioavailability and efficacy limiting their mainstream uses in treating gastroenteritis. The use of stable nano-particles that deliver plant-based medicine has attracted attention in the field of drug development. Such an approach could be an option for effectively delivering plant-based

medicines, safely to the lower gastro intestinal tract, the site of *Salmonella* and *E. coli* with the added benefits of controlled release at this site to increase bioavailability reducing required doses. Benefits would also include reduced side effects and higher efficacy of antidiarrheal compounds from medicinal plant extracts. This study then aimed at designing and characterizing the plant-based chitosan-modified poly (lactic-co-glycolic acid) nanoparticles for *Salmonella* and *E. coli*-induced gastroenteritis. Medicinal plants used as an alternative treatment for gastrointestinal infection were collected from Iikokola village in the Omusati region in Namibia. Organic and aqueous extracts were prepared from the collected plants, screened for the present bioactive compounds, and tested for their potential in eliminating multidrug-resistant clinical and reference strains of *Salmonella* and *E. coli* that are commonly associated with bacterial gastroenteritis. The antibacterial activity against clinical and reference strains of *Salmonella* and *E. coli* and cytotoxicity to human fibroblast cells (NIH/3T3) of prepared extracts were also determined. Plant extracts with potent antibacterial activity against clinical and reference strains of *Salmonella* and *E. coli* were encapsulated into poly (lactic-co-glycolic acid) nanocarriers coated with chitosan (CMPLGA) and characterized nanoparticles' morphology, size, zeta potential, polydispersity index, encapsulation efficacy, and pH based *in vitro* release in simulated gastrointestinal tract conditions. Furthermore, the stability of the designed nanoparticles was determined by monitoring the physicochemical properties, *in-vitro* cytotoxicity, and antibacterial activity of lysed and un-lysed nanoparticles stored at 4 and 25 ºC for 24 weeks.

As part of this study, an ethnomedicinal survey was conducted and 19 medicinal plants that are used in Iikokola village to treat gastrointestinal conditions and other diseases were

documented. Crude extracts of five of the frequently used medicinal plants used in this study (*Lantana camara, Terminalia sericea, Grewia tenax, Albizia anthelmintica,* and *Chorcorus trides*) were used to validate the phytochemical compositions of these plants and were found to contain multiple bioactive compounds. The collected plants showed potent antibacterial activity against multidrug-resistant clinical *Salmonella* and *E. coli* strains. Mild cytotoxicity to the mouse fibroblast cells (NIH/3T3) line was observed for the five plant extracts. All extracts showed an IC_{50} above 50 µg/ml. Encapsulating the selected plant extracts in CMPLGA enhanced the antibacterial properties of the plant extracts, reduced the cytotoxicity, and improved the *in vitro* release properties of the encapsulated extracts at pH 1.2 and 7.4. Lastly, factors such as lyophilization showed a change in the physicochemical properties of the formulated CMPLGA nanoparticles. Prolonged storage at 25 ºC tremendously reduces the efficacy of the CMPLGA nanoparticle, while storing at 4 ºC has only shown a minimal decrease in the efficacy of the CMPLGA nanoparticles. Overall, the use of CMPLGA nanoparticles enhanced the antibacterial activity of the plant extracts in this study and improved their *in vitro* release properties in simulations at a pH of 1.2 (stomach pH) and 7.4 (ileum pH). The use of CMPLGA nanoparticles also reduced the cytotoxicity of the extracts by over 20%. The toxicology and release properties of the formulated CMPLGA nanoparticles should be evaluated in an *in vivo* model as part of their development for mainstream use in treating *Salmonella* and *E. coli*-induced gastroenteritis.

LIST OF PUBLICATIONS

1. **Albertina M. N Shatri** and Davis R Mumbengegwi (2022). Ethnomedicinal use and phytochemical analysis of medicinal plants used to treat gastrointestinal conditions by Awambo people in Iikokola Village, Namibia. Scientific African. https://doi.org/10.1016/j.sciaf.2022.e01428.
2. **Albertina M. N Shatri** and Davis R Mumbengegwi (2022). Evaluation of antibacterial activities and cytotoxicity of selected medicinal plants used in the Omusati Region of Namibia, for management of gastroenteritis, against a panel of multidrug-resistant clinical and reference strains of *Salmonella* and *Escherichia coli*. South African Journal of Botany. (Manuscript under review).
3. **Albertina M. N. Shatri** and Davis R. Mumbengegwi. (2021). Ethnomedicinal uses phytochemical characterization, and antibacterial activity of *Grewia tenax* and *Albizia anthelmintica* extracts against multidrug-resistant pneumonia-causing bacteria. Journal of Pharmacognosy and Phytotherapy. 13(1), pp. 7-17. DOI: 10.5897/JPP2020.0601

LIST OF CONFERENCE AND POSTER PRESENTATIONS

1. **Albertina M.N. Shatri*,** Davis R. Mumbengegwi, Yolandy Lemmer. The effect of lyophilization on the physiochemical and biological properties of plant-based chitosan modified- PLGA nanoparticles. National Research Student Symposium. NIPAM. November 2022. Oral presentation.
2. **Albertina M.N. Shatri*,** Davis R. Mumbengegwi, Yolandy Lemmer. Nanomedicine: The future for oral delivery of phytomedicine targeting re-emerging multidrug-resistant pathogens. Showcase of the schools: Faculty of Health Sciences and Veterinary Medicine. The University of Namibia. July 2022. Oral poster (invited speaker).
3. **Albertina M.N. Shatri*.** Antibacterial activity of Namibian medicinal plants against clinical diarrheal strains from Namibia pediatric ward in Katutura State Hospital. NIP Research seminar, 3rd June 2022. Virtual (Invited speaker).
4. **Albertina M. N Shatri*.** Namibian medicinal plant extracts have the potential for use as complementary and alternative medicines for the treatment of multidrug-resistant clinical *Salmonella* and verotoxigenic *Escherichia coli 157:H7*. Executive Management Committee meeting of the African Institute in Indigenous Knowledge Systems (AIIKS)06 June 2022. Virtual (Invited speaker).
5. **A.M.N Shatri*** and D.R. Mumbengegwi. Namibian Medicinal Plant Extracts Have the Potential for Use as Complementary and Alternative Medicines for the Treatment of Multidrug-Resistant Clinical *Salmonella* and *Verotoxigenic Escherichia coli* 157:H7. Annual Research Conference. 2021.

6. **Shatri Albertina***, Lemmer Y, Kalombo L, Mandiwana V, Mumbengegwi D. R. (2021). Nano Carriers as A Potential Delivery System for Phytomedicines For Gastroenteritis. School of Public Health, Oshakati campus, Research Symposium (2021). (Oral presentation).
7. **A.M.N Iikasha*** and D.R. Mumbengegwi. (2019). Documentation of the ethnomedicinal uses of selected medicinal plants to treat gastrointestinal conditions and other ailments in Iikokola, Namibia. National Student Research Symposium. (Oral presentation).

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LIST OF ABBREVIATIONS AND/OR ACRONYMS

A. anthelmintica	*Albizia anthelmintica*
Ag	Silver
AIEC	Adherent invasive *E. coli*
ATP	Adenosine Triphosphate
Au	Gold
C. jejunum	*Campylobacter jejunum*
C. tridens	*Chorchorus tridens*
C.E	Clinical *E. coli*
C.S	Clinical *Salmonella*
CaCO2	Colorectal adenocarcinoma cells
CAM	Complementary Alternative Medicine
CDC	Center for Disease Control and Prevention
CMPLGA	Chitosan Modified PLGA
CMPLGA(C)	Chitosan Modified PLGA (*Chorcorus tridens*)
CMPLGA(EE)	Chitosan Modified PLGA (Empty Encapsulation)
CMPLGA(G)	Chitosan Modified PLGA (*Grewia. tenax*)
CMPLGA(LS)	Chitosan Modified PLGA (*Lantana camara* seed)
CMPLGA(LT)	Chitosan Modified PLGA (*Lantana camara* twig)
CMPLGA(T)	Chitosan Modified PLGA (*Terminalia sericea* seed)
DAEC	Differential Adherent *E. coli*
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DW	Distilled water
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDA	United States Food and Drug Administration
FIV	Family Importance Value
G. tenax	*Grewia tenax*
GI	Gastro-Intestinal
H. pylori	*Helicobacter pylori*
HCT-166	Human Colorectal Carcinoma cell line
HepG-2	Human liver cancer cell line
HPLC	High-Performance Liquid Chromatography
IC50	Half-maximal inhibitory concentration
IgA	Immunoglobulin A
L. camara	*Lantana camara*
MBC	Minimum Bacteria Concentration
M-cells	Microfold Cells
MDR	Multi-drug resistant
MIC	Minimum Inhibitory Concentration

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction

NCRST National Commission on Research Science and Technology

NiH/3T3 3-day transfer, inoculum 3 x 10^5 cells

Pa Particle diameter

Pb Particle degree

PBS Phosphate Buffer Saline

PCS Photon correlation Spectroscopy

Pdi Polydispersity index

PLGA Poly (lactic-co-glycolic acid)

PMLA Poly (malic acid)

PVA Polyvinyl acetate

R.E Reference *E. coli*

R.S Reference *Salmonella*

RFC Frequency of Citation

RNA Ribonucleic acid

rpm rotation per minute

S. Typhimurium *Salmonella Typhimurium*

SEM Scanning Electron Microscopy

SRB Sulforhodamine B

SS Stock Solution

STEC Shiga toxin-producing *E. coli*

T. sericea	*Terminalia sericea*
USFD	Unites States' Food and Drug Administration
UV	Use value
UV/Visible	UV/Visible spectroscopy
V. cholera	*Vibrio cholera*
WHO	World Health Organization

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DEDICATION

I dedicate this Ph.D. to the Infectious Disease Control and Drug Discovery and Development teams globally. To the Pediatrics unit in Katutura State hospital, Community members of Iikokola Village, Omusati region. To my family, our vision and prayers for a fruitful life are through the guides of Almighty God.

Al-ḥamdu l-illāhi rabbi l-ʿālamīn

DECLARATION

I, Albertina Mariina Ndinelao Shatri, declare that this study is a true reflection of my research and that this work or part thereof has not been submitted for a degree in any other institution of higher education.

Name of student: Albertina Mariina Ndinelao Shatri **Signature:** **Date:**

CHAPTER 1: INTRODUCTION

1.1 Background

Acute diarrhea is a condition of the gastrointestinal tract (GI) which is generally described as having three or more liquid or lose bowel movements per day (Riddle, DuPont, and Connor, 2016). Infectious diarrhea is caused by viral, bacterial, and parasitic pathogens (Li *et al.,* 2016). Bacteria such as *Escherichia coli* (*E. coli*), *Salmonella, Campylobacter,* and *Shigella* species threaten the lives of millions of people around the world (Chlebicz and Śliżewska, 2018). *Salmonella typhimurium* and *Salmonella paratyphi* cause 16 million morbidity and 600 000 mortality cases annually (Tala *et al.,* 2015). In Namibia, diarrheal diseases are among the top 10 causes of death (Centers for Disease Control and Prevention (CDC), 2020). In 2020 diarrhea was responsible for 17% of morbidity across all age groups in Namibia. In the Ohangwena region of Namibia specifically, diarrhea disease was reported to affect 19% of children under the age of 5 (Bauleth, Mitonga, and Pinehas, 2020).

Enteric infections are diagnosed utilizing bacterial cultures and microscopy to detect the presence of pathogens in the sample. Selective media allow a culture of specific *Salmonella, Shigella, E. coli,* and *Campylobacter* species (Pawlowski, Warren, and Guerrant, 2009). Isolation of cultured organisms in a clinical setting allows easy detection of the sensitivity to antimicrobial agents as well as identifying specific strains, virulence factors, or toxins during investigations of outbreaks. The polymerase chain reaction is also used and is found to be reliable although it is an expensive technique Scallan *et al.,* 2005).

Oral rehydration solution (ORS), zinc supplementation, antidiarrheal agents, and antibiotics are currently used in managing diarrhea (Riddle *et al.,* 2016). Nevertheless, treatment options such as ORS and Zinc supplementation do not cure diarrhea, but only reduce the severity of the diarrheal episodes and reduce dehydration, however, it is recommended as a first attempt to manage gastroenteritis (Riddle *et al.,* 2016). Antibiotics are used in cases of severe gastritis, especially in immunocompromised patients (Gotfried, 2022). However, antibiotics such as amoxicillin, ampicillin, ciprofloxacin, etc. used to combat *Salmonella* diarrheal infections are non-specific hence, they kill the microbial flora hence they should be taken with probiotics to protect the microbial flora (Orenstein, 2017).

Although there are several options for antimicrobial therapy for diarrheal diseases, their efficacy is limited because of the occurrence of drug-resistant bacteria. The global burden associated with drug-resistant infections assessed across 88 pathogens was approximately 4.95 million deaths in 2019 and *E. coli* is among the top 6 leading causes of death (Lancet, 2022). Many conventional medications have failed to show significant improvement in the overall survival of infected patients (Gao *et al.*, 2014). Hence at the 68th World Health Assembly in May 2015, the Global Action Plan (GAP) to tackle antimicrobial resistance was endorsed. In response to this call, Namibia established the Namibian Antimicrobial Resistance National Action Plan (NAAP). The overall aim of the plan was to ensure, for as long as possible, continuity of successful treatment and prevention of infectious diseases with effective and safe medicines that are quality-assured, used responsibly, and universally accessible to all who need them. The developed plan has key elements namely surveillance, prevention, antimicrobial use, awareness, collaboration, communication,

education, training, research, and development. Through the Namibian Institute for Pathology (NIP) the pathology and microbiology tests conducted suggest that there is increasing drug resistance in humans, including significant resistance to amoxicillin, the current first-line drug in the standard treatment guidelines (STGs) in Namibia for most bacterial infections. Moreover, there is a lack of established surveillance systems for drug resistance resulting in an incomplete picture of the true extent of the AMR problem in Namibia (The Namibian Antimicrobial Resistance National Action Plan, 2017). Additionally, until 2017, there have been no studies done or published on the disease burden of enteric diseases in Namibia. Active antibiogram surveillance programs are available only for animals. To date, there is still insufficient data on the antimicrobial resistance burden in Namibia despite the development of NAAP. Moreover, a study highlighted that the first-line antibiotic choices of doctors in Namibia are not informed by the Namibia Standard Treatment Guidelines and the local and regional antimicrobial sensitivity data (Pereko, Lubbe, and Essack, 2015). This can contribute to the emergence and re-emergence of resistant pathogens. Moreover, despite the agency of microbial resistance and higher statistics of microbial morbidity and mortality cases, there is limited data on the microbial resistance profile in Namibia (Iikasha *et al.,* 2019).

Comprehensive evidence-based guidelines for the management of acute gastroenteritis recommendations, prohibit the use of antibiotics when treating acute gastroenteritis in children (Bruzzese *et al.,* 2018). Irrational use of antibiotics results in prolonged symptoms contribute to antimicrobial resistance and increase the risk of complications of some infections such as hemolytic uremic syndrome associated with Shiga toxin-producing *Escherichia coli*, and prolonged shedding of *Salmonella* (Jones, 2017). Most

antibiotics are also not target-specific and their active compounds are metabolized in the stomach and which reduces their effectiveness (Rhee *et al.,* 2019). Furthermore, due to high antibiotic dosage intake, patients suffer from side effects such as nausea, severe diarrhea, and vomiting (Riddle *et al.,* 2016). The use of antibiotics in patients with diarrhea also results in direct toxic effects of antibiotics on the intestine which can alter digestive functions and cause pathogenic bacterial overgrowth leading to antibiotic-associated diarrhea (Song *et al.,* 2008).

Medicinal plants have been reported to be a better option compared to antibiotics and ORS for the treatment of diarrhea due to their long history of use with no reported side effects, abundance, and reported antibacterial and antidiarrheal properties (Akram *et al.,* 2020). In many communities, extracts, decoctions/concoctions, or ashes of various plant parts (roots, rhizomes, tubers, aerial parts, stem barks, and leaves) are used as remedies for diarrhea and other illnesses. Medicinal plants enriched with bioactive compounds such as tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids are reported to have anti-diarrhoeal and antibacterial activity (Njume and Goduka, 2012). It is estimated that over 50% of commercially available synthetic pharmaceutical products are derived from medicinal plants (Ozioma and Chinwe, 2019). Plant-derived anti diarrheal pharmaceutical products such as aesculetin used as an anti-dysentery from *Frazinus rhychophylla,* hemsleyadin used as anti-Bacillary dysentery from *Hemsleya amabilis,* and agrimophol an anthelmintic treatment form *Agrimonia supatoria* are currently prescribed and are sold by pharmaceutical companies. Over 80% of the population in developing countries make use of medicinal plants as a complementary or alternative treatment for communicable diseases such as diarrhea within their communities (Cheikhyoussef, Mapaure, and Shapi,

2011, Erkon, 2014). Most findings are limited only to the pharmacological application as well as the ethnomedicinal use of medicinal plants as remedies for diarrhea, hence there is a need for more research in this area (Njume and Goduka, 2012). Despite the potential of plant-derived antidiarrheal treatments such as berberine which can relax intestinal smooth muscle and prolong the residence time of intestinal contents to fully digest and absorb the intestinal contents to treat functional diarrhea as well as aesculetin, and neoadrographoid for treat dysentery and other diarrhea-like conditions (Njume and Goduka, 2012; Yu et al., 2020), most of the secondary metabolites in medicinal plants with antibacterial properties such as alkaloids, saponins, coumarins, and triterpenoids are metabolized in the stomach and this limits their efficacy. Moreover, most diarrheal medications are prepared in the form of decoctions that are reported to be less favorable due to the taste, prolonged preparation time, and limited shelf life after preparation (Yang and Ross, 2010). Therefore, despite their benefits reported in use within the traditional setting, the insufficient quality assurance and uncontrolled properties of the medicinal plant as antidiarrheal treatments limit their potential alternative options for treating diarrhea and other gastrointestinal conditions (Mensah *et al.*, 2019).

Recent advances in nanotechnology offer new hope for infectious disease treatment (Kirtane *et al.*, 2021). In modern-day medicine, nanotechnology and nanoparticles are essential tools in disease monitoring and therapy. The term “nanomaterials” describes materials with nanoscale dimensions and are broadly classified into natural and synthetic nanomaterials (Chenthamara *et al.*, 2019). Nanomedicine for delivering antidiarrheal medications provides an opportunity to improve the efficiency of the antibacterial regimen. Nanosystems that are used for targeted delivery of antibiotics have benefits over

current treatment options such as the ability to increase the increased solubility, improve stability, improved epithelium permeability and bioavailability, prolonged antibacterial treatment half-life, tissue targeting, and minimal adverse effects (Yeh *et al.,* 2020). The nanoparticles' sophisticated material engineering tailors the controllable physicochemical properties of the nanoparticles for bacterial targeting through passive or active targeting. Nanotechnology allows the development of drug carriers such as nano-capsules, nanoparticles, liposomes, micelles, and polymers for targeting different parts of the body (Laroui *et al.,* 2011). The use of nanoparticles helps to deliver a drug directly to the target site or site of action which reduces the dosage of drugs to be taken (Tewabe *et al.,* 2021). This leads to a decrease in common side effects associated with the current traditional methods that require the use of large dosages for effective treatment (Kothamasu *et al.,* 2012). Pathogenic bacteria maintain a negative surface charge under physiological conditions. Therefore, cationic nanoparticles capable of binding with bacteria via electrostatic interactions have been explored for effective bacterial targeting. This strategy is attractive for its multivalent effect and the ability to target poly-microbial infections. As a result, a diverse range of bactericidal polymers and peptides have been incorporated into various nanoparticle designs for antibacterial application (Gao *et al.,* 2014). Therefore, the approach of applying nanocarriers to deliver antidiarrheal extracts and medicinal compounds could enable an increase in efficacy, target specificity, multivalent effect, and the ability to target polymicrobial infections caused by *Salmonella* and *E. coli* while controlling the bioavailability of medicinal plant extract by limiting their bioavailability to the sites of infection (Yang *et al.,* 2020).

1.2 Statement of the problem

Despite the available treatment options for gastroenteritis, in Namibia, the morbidity rate of diarrhea was 17% in 2014 in all age groups and to date, diarrhea is responsible for 5% of all deaths in children under 5 years old. Moreover, in 2020, from a sample of 530 children in the Ohangwena region, 23.8% of children got diarrhea (Bauleth *et al.,* 2020, CDC, 2020). In a study conducted on children under 5 in Windhoek in 2018, *Salmonella Typhi* and *E. coli* were also responsible for most bacterial diarrhea cases reported in that study (Iikasha *et al.,* 2017). The available diarrheal antibiotics such as gentamycin, amoxicillin, erythromycin, and many other antibiotics used to kill diarrheal bacteria have shown higher patterns of resistance with many side effects. This is because large dosages should be administered for effective treatment when using these antibiotics. This makes them unsuitable for children and immune-compromised patients. In addition, antibiotics do not target specific pathogens hence they kill both pathogenic and beneficial microbiota found within the GI tract (Bruzzes, *et al.,* 2018). There is a long history of medicinal plants as alternative/complementary medicine for human ailments. Namibian medicinal plants have been reported to possess secondary metabolites which are linked to antidiarrheal properties of medicinal plants (Iikasha *et al.,* 2019). However, the ethnomedicinal use of most plants in Namibia to treat gastrointestinal conditions is not well documented. Moreover, there is the limited scientific knowledge to validate the ethnomedicinal uses of plants in Namibia to treat gastrointestinal conditions (Akmar *et al.,* 2020). To treat gastroenteritis, like antibiotics, most of the bioactive compounds are metabolized before they reach the site of action which reduces their bioavailability at the site of action, hence they too are taken in large dosages for effective treatment. Moreover,

the toxicity profile and prolonged side effects of many commonly used plants are not well documented. Despite the long history of using medicinal plants and their effectiveness in curing gastroenteritis in the traditional setting, more must be done to determine their safety and efficacy while improving the bioavailability at the site of infections (Moreira *et al.,* 2014; Kirtane *et al.,* 2021). Nanotechnology provides a suitable option that can enable the improvement of properties of promising plant-based medicine through targeted drug delivery. The use of stable target-specific- nanoparticles could be a possible way of delivering plant-based medicine safely to the site of bacterial infection with increased bioavailability. This will result in improved biophysics of medicinal plant extracts. Hence, the overall objective of this study was to design and characterize the plant-based Chitosan-modified Polylactic-co-glycolic acid (CMPLGA) nanoparticles for the treatment of *Salmonella* and *E. coli*-induced gastroenteritis. This was done to improve the biophysics of locally used plants to treat gastroenteritis in Namibia.

1.3 Aims and objectives of the study

To develop a plant-based drug delivery system based on medicinal indigenous knowledge, for treating gastroenteritis caused by *E. coli* and *Salmonella*.

1.3.2 Sub-objectives

1. To record the ethnomedicinal uses of plants to treat diarrhea in Iikokola village, Omusati region, Namibia.

2. To determine the qualitative and quantitative phytochemical compounds in extracts from selected medicinal plants identified in Iikokola village.

3. To determine the antibacterial activities of the selected plant extracts against clinical and reference isolates of *Salmonella* and *E. coli* and the *in vitro* cytotoxicity of the potent extracts to Normal mouse fibroblast cells (NIH/3T3) respectively.

4. To develop Chitosan-modified PLGA (CMPLGA) nanoparticles as carriers for antibacterial plant extracts and characterize their morphology, size, polydispersity index, encapsulation efficacy, and *in vitro* release in simulated gastric and intestinal fluids.

5. To determine the effect of lyophilization and storage temperature of the plant based CMPLGA nanoparticles on the stability, antibacterial activity, and *in vitro* cytotoxicity of the developed encapsulated antibacterial plant extracts to Normal mouse fibroblast cells (NIH/3T3).

1.4 Significance of the study

This study is important because it is the first to design plant-based CMPLGA nanoparticles for the delivery of plant extracts for treating *Salmonella* and *E. coli*-induced gastroenteritis. The incorporation of polymers to deliver plant-based phytomedicine with the potential to treat gastroenteritis will open up doors to exploring new methods of effectively delivering plant-based medicine with reduced dosage, improved bioavailability, and reduced side effects. This will further validate the existing ethnomedicinal knowledge of Namibian local plants that are documented in this study based on their uses to treat gastroenteritis in Namibia. The current data will further provide the Namibian perspective on treating gastroenteritis and this will help increase awareness of the significance of ethnomedicine as a complementary or alternative medicine for gastroenteritis. The knowledge gathered in this study will enable the comparison of this

knowledge with the ethnomedicine knowledge from other regions. The findings of this study showed that there is a difference in the efficacy of the extracts and antibiotics to clinical and reference strains, which offers known knowledge that the use of reference strains alone in screening the efficacy of pharmaceutical products is not enough, hence clinical isolates should always be used for reliable data when depicting efficacy or antibiotic resistance pattern. The findings of this study also prove the potential of chitosan and PLGA as suitable polymers for encapsulating phytomedicine. This finding will provide useful data to Namibian policymakers regarding the potential of medicinal plants as alternative /complementary medicine for gastroenteritis.

1.5 Limitations of the study

The Mouse fibroblast 3T3 cell line was used to determine the cytotoxicity of the plant extracts and the nanoparticles. This may not fully reflect the toxicity of the extract on the gastrointestinal tract, an epithelial cell line would have been more appropriate. Moreover, the cell-based assay may not fully reflect the antibacterial activity like an *in vivo* model. There may also be differences in the *in vivo* release profile of the nanoparticles since the simulation used in this study did not contain the pepsin enzyme, which is found in the stomach, hence there may be some differences in the mode of action and release profile of the developed drug delivery system.

1.6 Delimitations

The ethnomedicinal knowledge reported is only for knowledge holders identified in the Omusati region. The plant extracts were only screened for selected phytochemical compounds that are known to have antibacterial properties such as saponin, alkaloid,

flavonoid, triterpenoid, tannin, anthraquinone, and steroid. This study only used cell-based models to evaluate the uptake assay and toxicity of the formulation.

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CHAPTER 2: LITERATURE REVIEW

2.1 Bacterial gastroenteritis is a global health concern

Every 1 in 10 people suffers from foodborne diseases annually claiming over 33 million lives every year. Children are at higher risk of foodborne diseases. Gastroenteritis is a diarrheal disease characterized by an increase in bowel movement frequency with or without fever, vomiting, and abdominal pain. Gastroenteritis affects about 550 million annually of which 220 million are children under the age of 5 years (World Health Organization (WHO), 2018). Causes of gastroenteritis include bacterial, viral, fungal, and parasitic, but this review will focus on bacterial causes. The common causative agents of bacterial gastroenteritis include *Shigella, Salmonella, Campylobacter, Yersinia, Vibrio cholera,* and *E. coli*. This study will focus on *Salmonella* and *E. coli* as the major causes of gastroenteritis globally (Sattar and Singh, 2021).

Patients with gastroenteritis normally present with clinical symptoms such as abdominal pains, nausea, diarrhea, and vomiting. The onset of disease symptoms occurs 6 to 72 hours after ingesting food contaminated with bacteria and the illness lasts 2 to 7 days (Khan, 2014). Some individuals become chronic asymptomatic carriers of diarrheal pathogens due to their ability to shed large numbers of bacteria in their feces and can subsequently transmit the pathogen to other people by contamination of food and water sources (Kurtz et al., 2017).

Since *Salmonella* and *E. coli* are foodborne pathogens, everyone is at risk of contracting gastroenteritis. However, in a healthy individual, gastroenteritis is self-limiting and would

not require the administering of antibiotics. On the contrary in the young, the elderly, or immunosuppressed patients, *Salmonella* or *E. coli* infections can lead to severe complications which may result in death (Malik, 2017). This is because these pathogens can spread systemically following the rupture of the gastrointestinal tract and internalization within phagocytes.

2.2 Important *Salmonella* and *E. coli* serotypes causing gastroenteritis and tailor the section to only those that cause diarrhea in humans.

Human salmonellosis can be categorized as either typhoid fever or nontyphoidal (Xu *et al.*, 2021). *Salmonella* genus is divided into two species namely *Salmonella bongori* and *Salmonella enterica* which are made up of, more than 2500 serotypes (WHO, 2018). Figure 1 shows the nomenclature of *Salmonella*. The most-reported mortality and morbidity cases in humans and warm-blooded animals are due to *Salmonella enterica* (Santos, 2014). *S. Typhimurium* and *S. enteritidis* are the main causative agents for acute gastroenteritis (Santos, 2014; Khan, 2014).

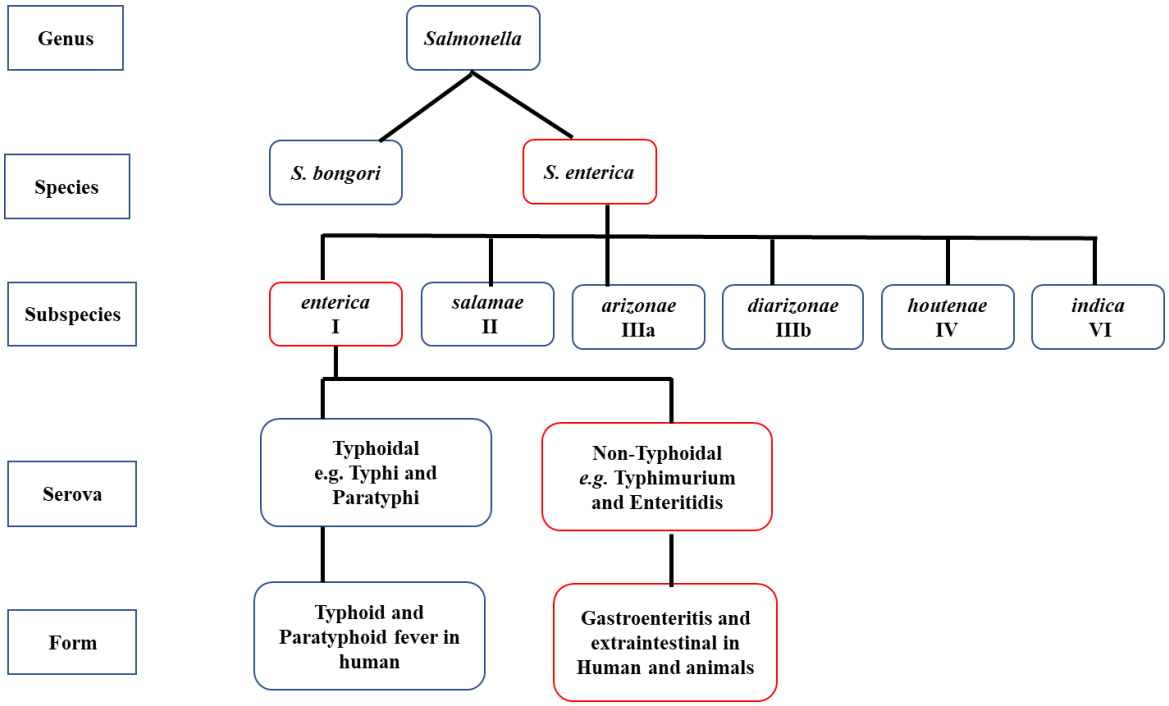

Figure 1: The nomenclature of *Salmonella* highlighting specie and Serova causing gastroenteritis in humans (Shatri, 2023).

Escherichia coli has been recognized as a cause of gastroenteritis using at least 2 main different pathogenic mechanisms such as direct invasion of the intestinal mucosa and production of either heat-labile or heat-stable enterotoxin (Mare *et al.*, 2021). Human infections with *Escherichia coli* O26:H11 and O26:H-untypeable (H-) or H-nonmotile (NM) strains are associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Durso, Bono, and Keen, 2005). Depending on virulence factors and phenotypic traits *E. coli* O26 is classified as enterohemorrhagic (EHEC) or enteropathogenic (EPEC). The *E. coli* O26 serotype was first reported to cause pediatric EPEC diarrhea (Gomes *et al.*, 2016). The enterotoxigenic group is made up of 4 sub-groups namely, enterohaemorrhagic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, and diffusely adherent *E. coli*. Enterotoxigenic *E. coli* (ETEC) and enteropathogenic

E. coli (EPEC) produce non-inflammatory diarrhea, whereas enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAggEC) produce inflammatory diarrhea (Hart *et al.*, 1993). While diarrheagenic *E. coli* strains are among the most common etiologic agents of diarrhea the enteropathogenic *E. coli*, and enterotoxigenic *E. coli* are Vero-toxin-producing, or Shiga toxin-producing (Jafari, Aslani, Bouzari, 2012). In a study conducted on Namibian children, *E. coli* 157:H7 VTEC was found to be responsible for *E coli*-induced gastroenteritis (Iikasha *et al.*, 2022).

2.3 Salmonella and *E. coli* host invasion pathways

Host infections are usually initiated by the ingestion of food or water contaminated with the bacteria. The acid pH of the stomach induces an acid tolerance survival response in *Salmonella*, causing physiological changes within the small surviving *Salmonella* subpopulation (Khan, 2014). *Salmonella* invades the small intestine where it is exposed to bile salts, enzymes, antimicrobial peptides, and secretory immunoglobulin A that are found within the lumen of the small intestine. *Salmonella* invades the small intestine by either colonizing M-cells and other intestinal epithelial cells, by CD18-expressing phagocytes, or across a damaged intestinal barrier (Khan, 2014).

Salmonella and *E. coli* travel through the mucous layer to invade intestinal epithelial cells such as the M-cells and dendritic cells (Khan, 2014; Govindarajan *et al.*, 2020). The attachment of the bacteria to the target specialized M-cells is initially promoted by fimbriae present on the bacteria's surface. M-cells are located on top of the lymphoid follicles of Peyer's patches whose role is to facilitate a pinocytotic sampling of luminal antigens required for mucosal immunity (Mabbott *et al.*, 2013). As the bacteria invade the

M-cells, it induces membrane ruffles, which surround the pathogen leading to endocytosis (Miller *et al.,* 2007). M-cells will then transport the bacteria across the epithelial barrier (Wang *et al.,* 2014). *Salmonella* can also use an alternative strategy to spread from the gastrointestinal tract by being taken up by CD18-expressing phagocytes that transmigrate across tissue barriers and transport Salmonella from the lumen of the gastrointestinal tract to the systemic circulation (Hallstrom and McCormick, 2011). After phagocytosis, *Salmonella* manipulates the function of the host cell to interfere with antigen processing and presentation (Khan, 2014). *Salmonella* can also disrupt tight junctions between adjacent epithelial cells, which normally help prevent leakage of water, ions, nutrients, and immune cells from the gastrointestinal tract (Awad, Hess, and Hess, 2017).

The mode of entry of *Salmonella* in the gut lumen varies according to the type of cell encountered on the gut epithelium. The M cells take up the bacteria utilizing receptor-mediated endocytosis. Dendritic cells on the other hand engulf *Salmonella* by phagocytosis. The membrane of epithelial cells is modified by the action of SPI1 to facilitate the entry of bacteria (Awad *et al.,* 2017). Once inside the gut lumen, *Salmonella* is then taken up by macrophages, T cells, B cells, and neutrophils figure 2 summarized the most common macrophage uptake (Shatri, 2021). Salmonella Typhimurium infects and survives within macrophages by adhering to the proliferating cell, invading, and surviving within the cells, ultimately resulting in the death of host cells in many cases (Gog *et al.,* 2012).

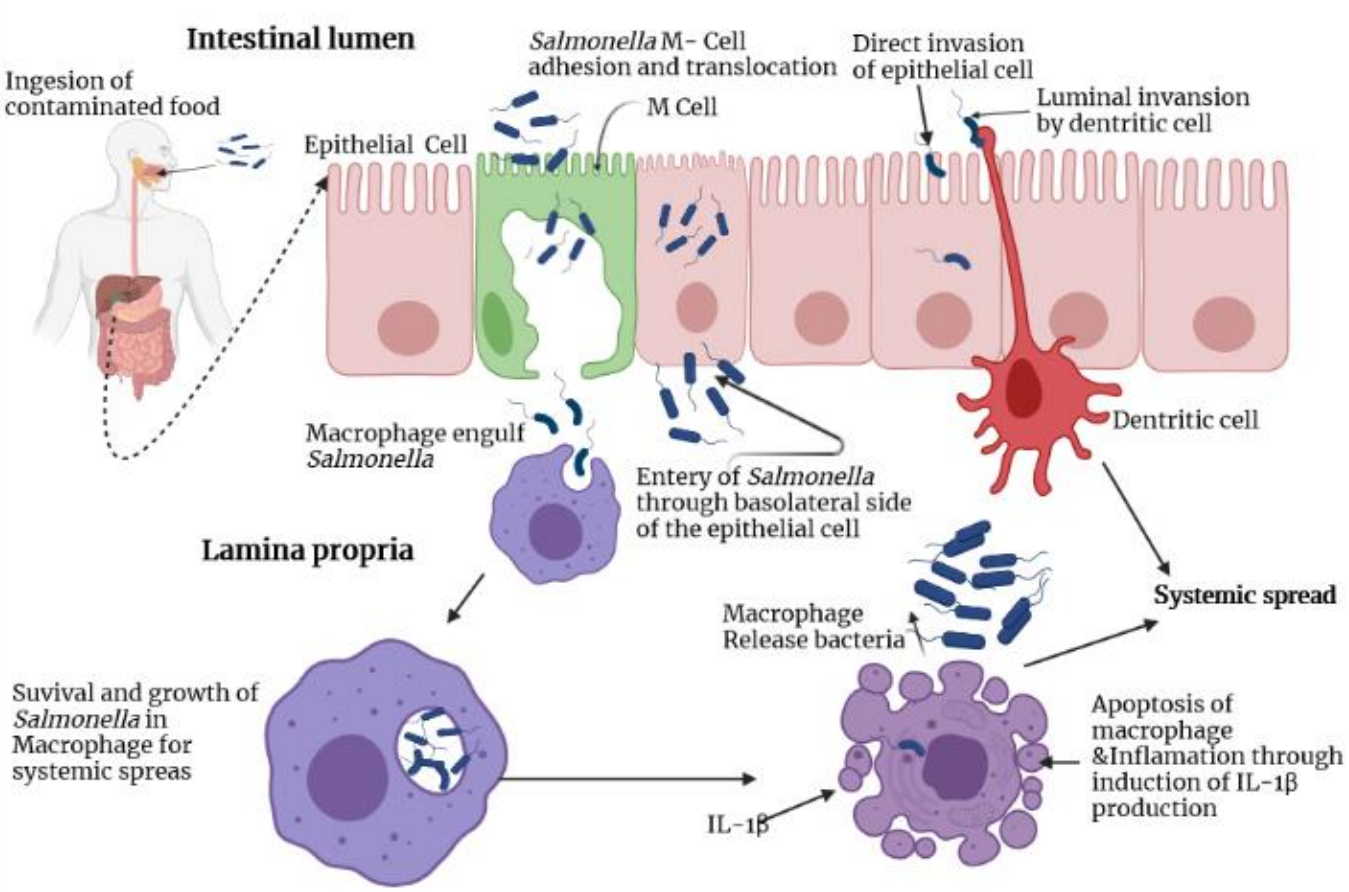

Figure 2: Breaching of gut epithelia by different *Salmonella* serotypes. Created with BioRender.com (Shatri, 2021).

There are several strains of *E. coli,* and they use different mechanisms when adhering to the host epithelium. Enteropathogenic *E. coli* (EPEC) and Shiga toxin-*producing E. coli* (STEC) are extracellular bacteria that form A/E (Attaching and Effacing) lesions as they attach to the intestinal epithelium and efface microvilli. Due to the presence of bundle-forming pili, EPEC can form micro-colonies, resulting in a localized adherence pattern (Mare *et al.*, 2021; Durso, Bono, and Keen, 2005). Enterotoxigenic *E. coli* (ETEC) uses colonization factors for attachment to host intestinal cells, while Enteroaggregative *E. coli* (EAEC) forms biofilms on the intestinal mucosa by adhering to each other as well as to

the cell surface forming an aggregative adherence pattern called a stacked brick. Diffusely adherent *E. coli* (DAEC) on the other hand forms a diffuse adherence pattern by dispersing over the surfaces of intestinal cells. Adherent invasive *E. coli* (AIEC) colonizes the intestinal mucosae of patients with Crohn's disease and is capable of invading epithelial cells as well as replicating within macrophages (Cepeda-Molero et al., 2017). AIEC uses type I pili to adhere to intestinal cells and long polar fimbriae that contribute to invasion. Enteroinvasive *E. coli* (EIEC) are intracellular bacteria hence they penetrate the intestinal epithelium through M-cells to gain access to the submucosa (Mare *et al.*, 2021; Bhunia, 2018; Hart, 1993). EIEC escapes submucosal macrophages by induction of macrophage cell death followed by a basolateral invasion of colonocytes and lateral spread as summarized in Figure 3 (Shatri, 2021).

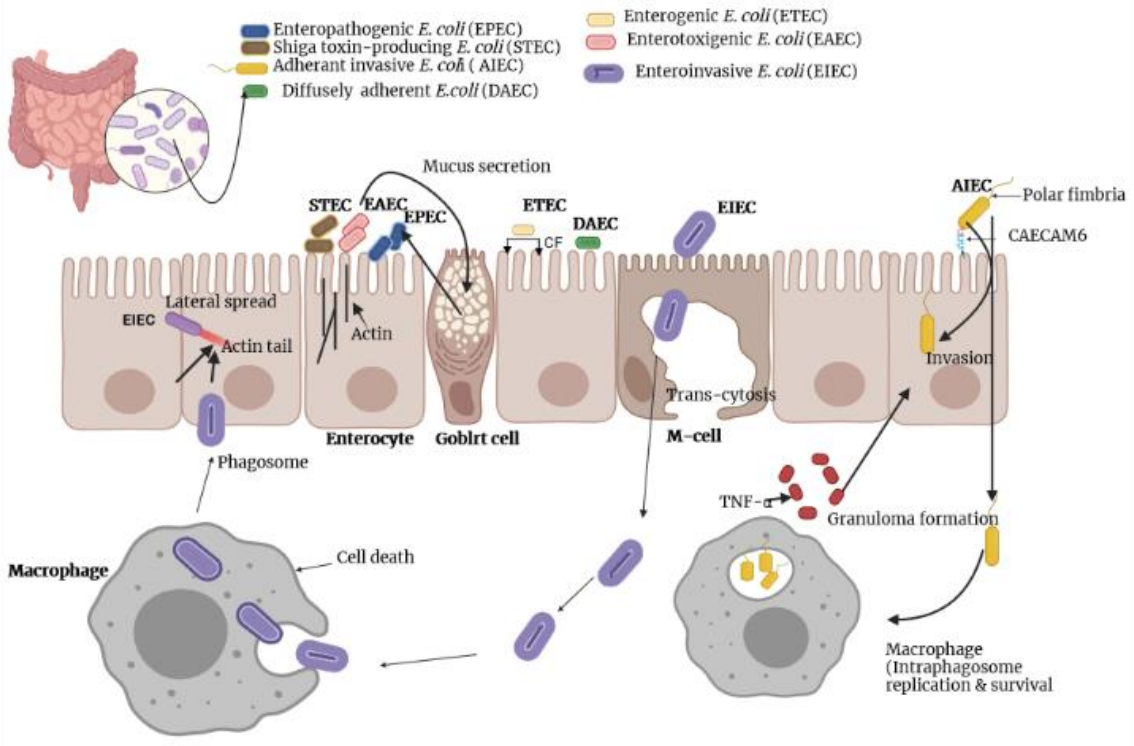

Figure 3: Breaching of gut epithelia by different *E. coli* serotypes. Created with BioRender.com (Shatri, 2021).

2.4 Management and treatment options for *Salmonella* and *E. coli* gastroenteritis

About 88% of diarrhea-associated deaths are attributable to unsafe water, inadequate sanitation, and insufficient hygiene (Ngogo *et al.,* 2020; CDC, 2019). Prevention is better than cure is important, especially for infectious diseases. Gastroenteritis can be avoided by ensuring people have access to clean water and that the community is trained on how to protect themselves from diarrhea (CDC, 2019).

Allopathic ways of managing diarrheal symptoms for gastroenteritis include the use of oral rehydration solutions for electrolyte replacement and rehydration. Infants, the elderly,

and immunocompromised patients may need to receive antimicrobial therapy for gastroenteritis. However, healthy individuals with mild or moderate gastroenteritis do not need to use antimicrobial therapy, since antimicrobials may not eliminate the bacteria, and this may result in antibiotic resistance (WHO, 2018). Antibiotics such as cephalosporin and fluoroquinolones are used as first-line treatment options for salmonellosis in adults while azithromycin is recommended for children. Moreover, ceftriaxone is also used as an alternative first-line treatment agent (CDC, 2019). Based on the Namibian treatment guidelines, the use of ORS is strongly encouraged to avoid dehydration and to provide the body with lost electrolytes. Zinc supplementation is also recommended for children. The use of antibiotics for acute bacterial gastroenteritis is not encouraged (Namibia standard treatment guideline, 2021).

Continuous uses of antibiotics have however caused *Salmonella* species to develop resistance to ceftriaxone, ciprofloxacin, ceftriaxone, or other antibiotics including ampicillin, chloramphenicol, fosfomycin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline (Nair, Venkitanarayanan and Johny, 2018; CDC, 2019; DiMarzio *et al.*, 2013). While there is limited data on the global trends' studies have been conducted in different parts of the world to show the antibiotic-resistant trends showing over 15% resistance patterns to antibiotics such as ampicillin, chloramphenicol, fosfomycin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole (Foley *et al.*, 2008; Eltai *et al.*, 2020). Moreover, since most conventional antibiotics lack pathogen target specificity, sometimes they kill both good and bad bacteria in the gut. The uncontrolled drug release limits the effectiveness of different antibiotics since most viable

compounds are metabolized in the stomach and only a few active ingredients reach the small intestine where salmonella resides (Upadhyay, 2014). Moreover, due to the high dosage used for effective treatment, side effects such as vomiting, severe diarrhea, and abdominal pain are associated with antibiotics (Riddle, DuPont, and Connor, 2016).

In a study of 88 participants of which 61 were asymptomatic and 27 were symptomatic of gastroenteritis, 85.2% (52/61) of the isolates from asymptomatic patients and 85.2% (23/27) of symptomatic patients have shown MDR to 26 antibiotics (Xu *et al.*, 2021). Recent reports also indicate decreased susceptibility of *S. Typhi* to fluoroquinolones, especially ciprofloxacin (Rahman *et al.*, 2014). Chloramphenicol has been suggested as the first-line therapy for enteric fever in many studies. Studies that describe the trends of antimicrobial susceptibility of *S. Typhi* and *S. paratyphi* A causing bacteremia in children and report therapeutic failure to ciprofloxacin and evaluate the possible use of chloramphenicol, ampicillin, ciprofloxacin, and third generation cephalosporins as first-line therapy in the treatment of enteric fever in children. The microbial susceptibility testing from Awol, Reda, and Gidebo (2021) revealed that all six of the *S.typhi* isolates showed sensitive to ceftriaxone and all 6 isolates showed resistant to nalidixic acid and cefotaxime. Multidrug resistance was observed among most of the isolates (Awol, Reda, and Gidebo, 2021). Figure 4 summarized most challenges faced with the oral delivery of most drugs. A study by Mohsen, Dickinson, and Somayaji, (2020) also highlights other side effects associated with antibiotics. While most of the reports on antibiotics focus on the benefits, limitations such as allergic reactions, vomiting, severe diarrhea, anaphylaxis, skin rash, headache, thrombophlebitis, drug induced-anemia, hematuria, dizziness, paralysis, etc. are normally not reported.

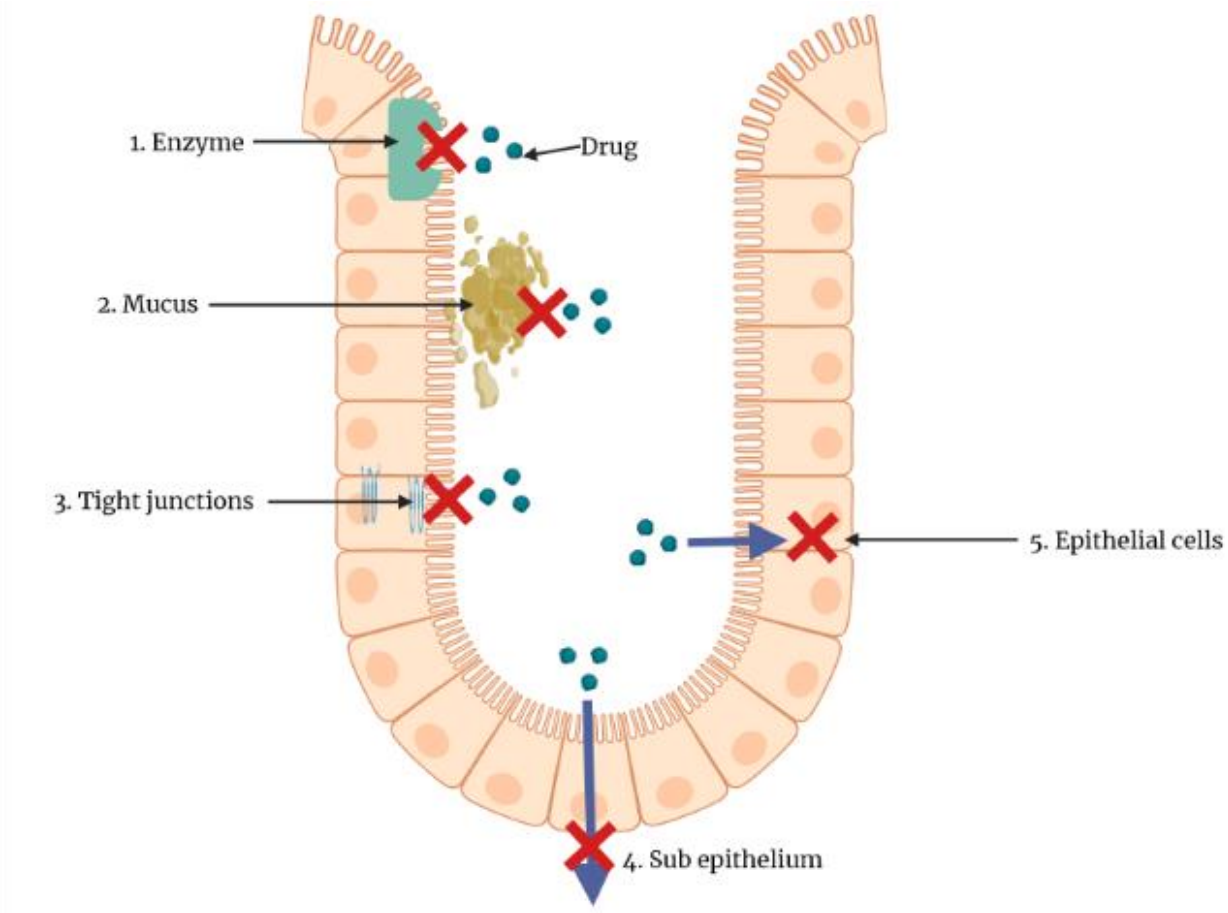

Figure 4: Common barriers to oral delivery of most diarrheal drugs: 1. the intestinal enzymes, 2. the mucus barrier, 3. tight junctions, 4. intestinal epithelial cells lining the gastrointestinal tract, 5. sub epithelial tissue. Created with BioRender.com (Shatri, 2022).

2.5 The use of medicinal plants as alternative treatment options for gastroenteritis

For centuries, the world population had relied on the use of medicinal plants for treating different ailments (Petrovska, 2012). It has been reported that between 75% and 80 % of the population in developing countries rely on medicinal plants as a primary health care

option, especially in rural communities (Pan *et al.,* 2014). In a rural community, people are faced with the challenge of poor sanitary conditions and limited access to clean drinking water. Moreover, there are also limited health services hence people opt for self-medication using medicinal plants which are often only available and affordable to people with limited recourses (Rakotoarivelo *et al.,* 2015). Medicinal plants are not only used as regional traditional treatments but countries such as India, Iraq, Iran, and China are also registered as official medicines that are verified with pharmacopeias (Ahmed, 2016).

In Namibia there is limited documented knowledge on the uses of plants for the treatment of gastrointestinal conditions hence there is a concern about losing this knowledge since, in most communities, the knowledge is transmitted orally from one generation to the next among traditional health practitioners (Iikasha, Bock and Mumbengegwi, 2017, Iikasha, Quaye and Mumbengegwi, 2020). Moreover, there is limited information on the safety and efficacy of natural products in Namibia and many other developing countries which limits their registration, regulation, and recognition of their clinical, pharmaceutical, and economic value as effective treatment options (Ahmed, 2016). Medicinal plants have additionally been a success in drug development and over 50% of the best-selling prescription drugs in use currently are derived from medicinal plants (Abdela, 2019).

Medicinal plants play a major role in pharmacological research and drug development, not only when plant constituents are exploited directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Shatri and Mumbengegwi, 2021). These bioactive compounds are generally used as the plant's defense mechanisms against insects, herbivores, and microorganisms. The wide variety comes from the plant's ability to synthesize a large arsenal of aromatic

compounds and their oxygen-substituted derivatives (War *et al.,* 2012). Plants are used for treating gastroenteritis and other infectious diseases due to the presence of bioactive compounds that are naturally present in plants (Iikasha *et al.,* 2020). Table 1 shows common bioactive compounds with antidiarrheal and antibacterial properties. Plant extracts with phytochemicals are reported to have the potential resistance-modifying agents with multiple modes of action such as modified target sites, inhibiting bacterial enzymes that inactivate antibiotics, increasing bacteria membrane permeability, and inhibiting efflux pumps. These all help to restore the effectiveness of antibiotics when combined with plant extracts (AlSheikh *et al.,* 2020).

Table 1: List of bioactive compounds with antidiarrheal and antibacterial against diarrheal activity

Class	Bioactive compound	Best extracting solvents	Activity	Mode of action	Reference
Phenolic compounds	Flavonoids tannins phenolic acids	ethanol, methanol, acetone, and ethyl acetate	antibacterial, antidiarrheal	1. Enzymes inactivation by the oxidized compounds through reactions with proteins via SH-groups.	Do *et al.,* 2014, Aslam and Janbaz 2019; Konate *et al.,* 2015.
Alkaloids	quinolones, metronidazole, squalamine	ethanol, methanol	antidiarrheal, antibacterial	1. Affecting cell division by inhibiting the activity of dihydrofolate reductase required to produce pyrimidine and purine precursors for amino acids, RNA, and DNA biosynthesis. 2. Respiratory inhibition and enzyme inhibition in bacteria. 3. Bacterial membrane disruption. 4. Inhibition of virulence factor.	Othman, Sleiman, Abdel-Massih, 2019; Truong *et al.,* 2019
Saponins	Triterpenoid, steroid	ethanol, methanol, acetone, ethyl acetate, dichloromethane	antidiarrheal, antibacterial	1. Surface-active properties enabling saponin to insert into the lipid bilayer of bacteria. 2. Bind to cholesterol causing cell lyses. 3. Disturb the permeability of the bacterial outer membranes' lipopolysaccharides causing an increase in cell permeability.	Asrie *et al.,* 2016; Abdela, 2019; Arabski *et al.,* 2012; El Aziz *et al.,* 2019

Anthraquinones	physcion, chrysophanol, aloe-emodin, rhein, sennosides	methanol, ethanol, chloroform	antibacterial, antidiarrheal	1. Spasmolytic activity mediated through Calcium ion channel blocking action. 2. Increases the permeability of the cell envelope leading to the leakage of cytoplasm and the deconstruction of the cell.	Aslam *et al.,* 2019; Cao and Zhao, 2011; Wei *et al.,* 2015.
Coumarins	umbelliferone, aegelinol, agasyllin	water,	antidiarrheal, antibacterial, antibiofilm	1. Reduce intestinal transit 2. Damaging the bacteria cell membrane.	da Cruz *et al.,* 2020; Yang *et al.,* 2016; Lončar *et al.,* 2020, Venugopala, Rashmi, and Odhav, 2013

2.6 Challenges of medicinal plant extracts within the gastrointestinal tract

Medicinal plant extracts for treating *Salmonella* and *E. coli*-triggered gastroenteritis should go through the stomach, intestinal lumen, the mucus membrane coating the intestinal epithelium, and finally the M cells of the epithelia and partially go systemic itself for effective eradication of these pathogens. The inside of the stomach is composed of four layers; from the innermost layer to the outermost layer, these are the mucosa, submucosa, muscularis externa, and serosa. The stomach is lined by a mucous membrane that contains chief cells that secrete gastric fluid (Hua, 2020). The intestinal epithelium is made up of villi that increase the surface region available for plant extract absorption however, the presence of absorptive enterocyte cells and mucus-secreting goblet cells covering the villi, tight junctions blocking paracellular passage, the epithelial cells of the gastrointestinal tract (Lundquist and Artursson, 2016), and the subepithelial tissue presents physiological properties of the GI tract cause poor extract absorption and availability due to low mucosa permeability and extract degradation before absorption (Dey, Paul, and Dhar, 2017). Figure 5 summarizes the barriers and challenges to the efficient oral delivery of plant-based medicine (Kyriakoudi *et al.*, 2021).

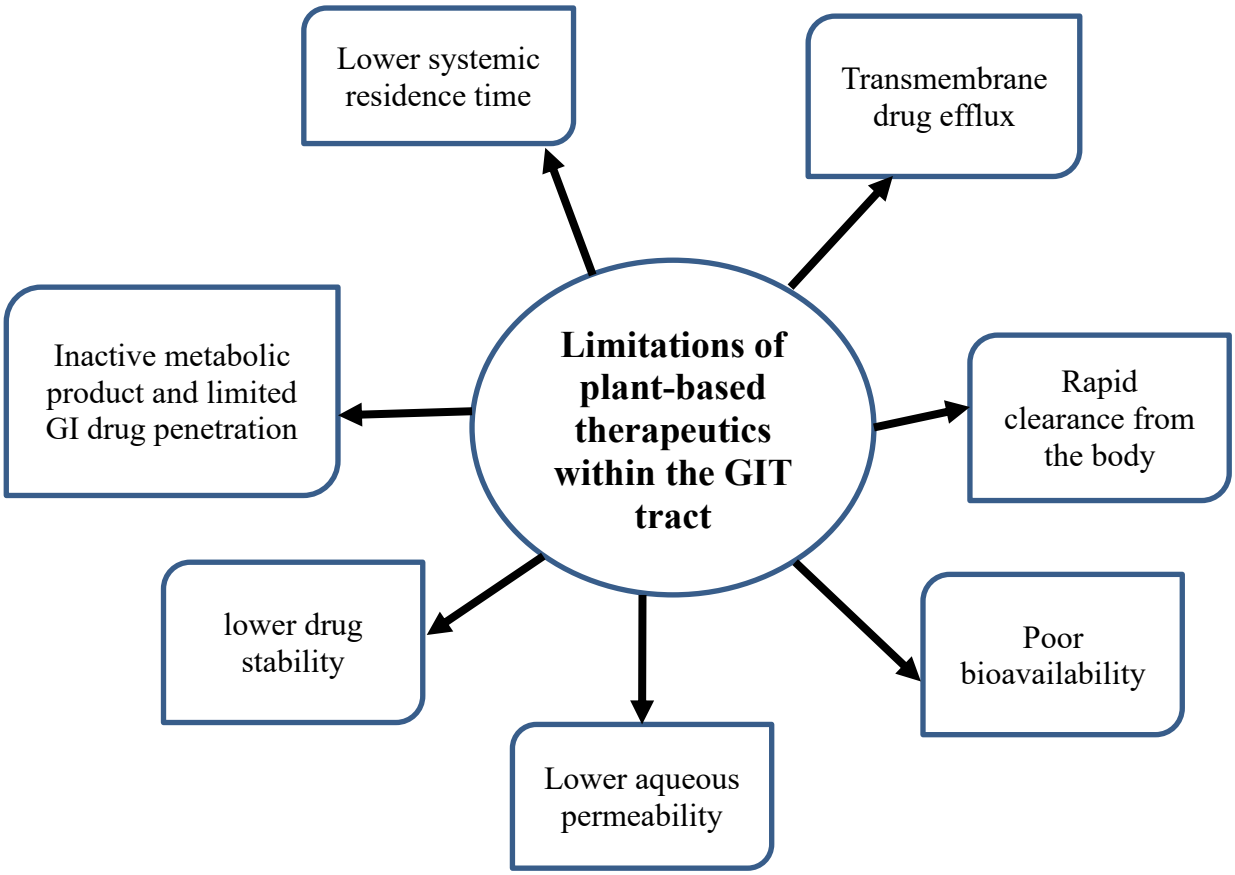

Figure 5: Major limitations of the plant-based therapeutic within the GI tract

2.7 Advances in oral drug delivery for regional targeting when treating gastrointestinal conditions

The oral route is the most common route of drug administration and can be used for both systemic drug delivery and for treating local gastrointestinal (GI) diseases (Kim and De Jesus, 2021). The gastrointestinal tract is divided into the upper GI tract which is made up of the mouth, pharynx, esophagus, stomach, and duodenum as well as the lower GI tract which is made up of the jejunum and ileum, cecum, colon, and rectum. *Salmonella* and *E. coli* reside within the Ileum and initiate diarrhea while colonizing and multiplying within the M-cells.

Oral delivery is the most preferred route for treating gastroenteritis, due to its advantages, such as ease of use, non-invasiveness, and convenience for self-administration. Moreover, nanoparticles can be designed with properties that enhance drug delivery to specific regions in the upper or lower gastrointestinal tract (Hua, 2020). Drugs administered via the oral route, however, generally have slower absorption, which is not preferred during an emergency. They might also be unpleasant in taste, cause gastric irritation, and/or undergo first-pass drug elimination processes in both the intestine and liver. In addition, the physiological environment in the GI tract can also affect the stability and solubility of drugs.

Gastroprotective drug delivery helps to extend the duration of the drug within the gastrointestinal tract while providing targeting site-specific drug release in the gastrointestinal tract (Vrettos, Roberts, and Zhu, 2021). This can be achieved using 4 types of delivery systems namely: 1. High density (sinking) system or non- floating systems which involves creating a drug with a density higher than that of normal stomach content. Iron powder and barium sulfate are used to develop the floating system. However, the effectiveness of this system in not yet approved for use in humans (Yaday *et al.*, 2021). 2. Floating drug delivery systems involve creating a drug that can maintain specific gravity lower than gastric contents. This system is desirable for drugs with an absorption window in the stomach or the upper small intestine and it increases the bioavailability of the drug and gastric retention period. Lower-density fatty materials or oils, or foam powder are used to develop this system. However, there are higher chances of drug sticking together or being obstructed in the gastrointestinal tract which may irritate (Ramanathan *et al.*, 2018). 3. non-effervescent systems are prepared using gel-forming or highly swellable

cellulose such as hydrocolloids, and polysaccharides, forming polymers such as polyacrylate, polycarbonate, and polystyrene. This approach creates hydrocolloids which can form contact with gastric fluid after oral administration and maintain lower integrity/ density when within the gastric environment (Ayalasomayajula *et al.*, 2020). 4. Alginate beads system which is developed using calcium ion, methoxylated pectin, and sodium alginate. With this system, sodium alginate solution is dropped into an aqueous solution of calcium chloride and causes the precipitation of calcium alginate after freeze dying a porous floating system is formed. This can increase gastric retention for up to 5.5 hours (Segale *et al.,* 2016). 5. Mucoadhesive drug delivery systems that can enhance drug absorption in a site-specific manner. The drug attaches to the mucosal surface and some of the materials used are polyacrylic acid, chitosan, cholestyramine, sodium alginate, hydroxypropyl methylcellulose, and polylactic acids which are used in developing modern nanoparticles for targeted drug delivery (Zhang *et al.,* 2021). Among the different delivery systems mucoadhesive drug delivery is extensively studied and being evaluated as carriers for targeted drugs (Patra *et al.*, 2018).

2.8 Nanotechnology and treatment of gastrointestinal conditions

Nanotechnology and its application have made a significant impact on different divisions of medicine and have become a useful tool for disease diagnoses and the development of treatments (Ibrahim, 2020). Advances in nanotechnology have developed better approaches that could help with disease detection, prevention, and treatment. Developing therapeutics based on the pathophysiology of gastrointestinal diseases can help with providing hope for future generations (Meganck *et al.*, 2021).

Nanoparticles are solid particles with a size range between 10 nm and 1000 nm (Khan *et al.,* 2014). Nanoparticle vectors have shown the potential to deliver drugs specifically and exclusively to regions of the gastrointestinal tract affected by the disease (Jain and Thareja, 2019). This approach can result in a prolonged significant reduction of the side effects of existing otherwise effective treatments such as antibiotics. Moreover, nanomedicine is a promising method that can offer a controlled release for gastrointestinal medicine (Salapa *et al.,* 2020). The gastrointestinal tract is a site of therapeutic absorption hence this makes it a favorable target system for nanotechnology applications (Ahmad *et al.,* 2012). The ability to regulate the behavior of nanomedicine through the digestive tract can result in successful treatment. The ability of nanomedicine to successfully interact with tissues in the gut highly depends on particle size, size distribution, morphology, hydrophilic-hydrophobic balance, and surface functionalization of the nanomaterial (Lombardo, Kiselev, and Caccomo, 2019).

The therapeutic potential of nanoparticles depends on their availability at the target site, the amount present, and the duration before degradation. Targeted drug delivery of gastrointestinal therapeutic compounds helps minimize the exposure of the drug to non-target tissues and this can help reduce side effects. The application of drug delivery systems for specific gastrointestinal conditions can improve drug efficacy and safety. This can be amplified by modifying the pharmacokinetic properties of the nanoparticles with the entrapped drug (Salapa *et al.,* 2020).

Most nanoparticles are made of biodegradable substances; therefore, they can easily be broken down into the GI tract. Manipulating nanoparticles' properties by altering the surface charges and particle size and other properties of nanoparticles will allow passive

and active drug targeting (Jain and Thareja, 2019). Unlike chemically modified conjugates, greater drug encapsulation can be obtained into the nanoparticles without any chemical interaction allowing full drug activity maintenance. Outer surface modification of the nanoparticles can allow a change in the bio-distribution of therapeutics. Target specificity can be obtained easily by anchoring specific surface structures on the nanoparticles as well as by magnetic-based targeting. Moreover, due to their smaller size, nanoparticles can penetrate through smaller capillaries; hence, allowing optimal dug deposition at the target site. Targeted drug delivery certifies that drugs are delivered to the site of interest, whilst minimizing the damage to the surrounding tissue (Poovi and Damodharan, 2018). They also allow a lower dose of the drug to be used, further preventing interaction with off-target sites, and improving patient quality of life (Patra *et al.*, 2018). Figure 6 shows a summary of the benefits of nanomaterials for the development of efficient gastrointestinal treatment (Deng *et al.*, 2022; Zhou and McClements, 2022; Fey *et al.*, 2022).

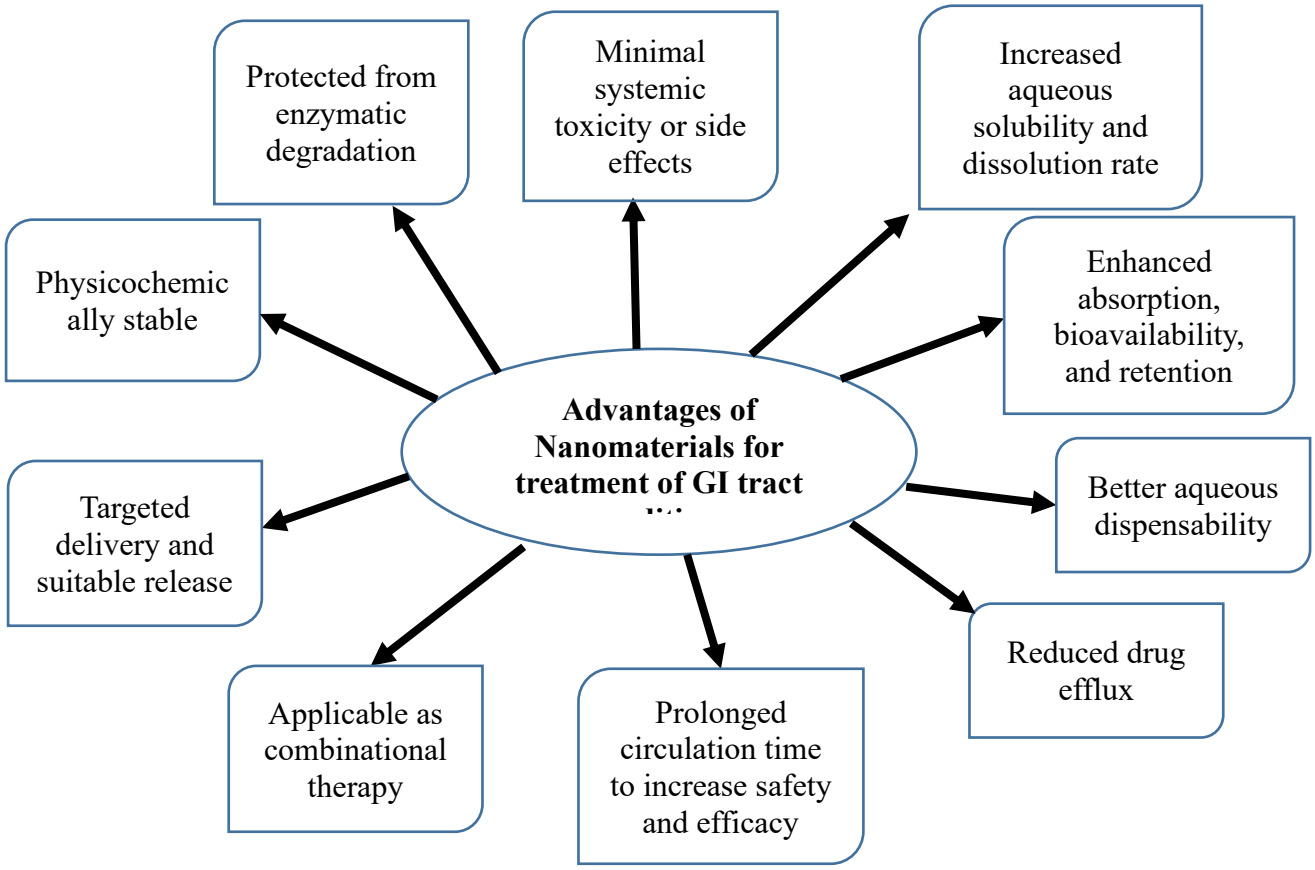

Figure 6: Benefits of nanomaterials for the development of efficient gastrointestinal treatment

2. 9 Types of nanoparticles applicable for studying the delivery of pure plant bioactive compounds, extracts, and essential oils with antibacterial

Various materials can be used to design nanoparticles to target different bacteria within the GI tract. For GI tract drug delivery, the nanoparticulate core or shell can load several payload drugs. Properties such as nanoparticle shape, size, and surface charge can be modified based on the targeted organ or cells as well as on material types, contents, and preparation processes for optimizing drug release (Attia *et al.*, 2019; Vega-Vásquez *et al.*, 2020). Among existing classes of nanoparticles, lipid-based, inorganic metal, polymeric, micellar, silica, and cell membrane-coated nanoparticles are the commonly studied nanosystems for antibiotic drug delivery (Yeh *et al.*, 2020). The bacterial killing

mechanisms of nanoparticles include membrane disruption, reactive oxygen species (ROS) production, ATP depletion, and DNA synthesis inhibition (Slavin *et al.*, 2017). These classes of nanoparticles help improve the solubility, stability, controlled release, and bioavailability of entrapped plant extracts (Shubhika, 2013). Examples of nanoparticles used to deliver plant-based compounds are given in sub-theme 2.9. 1 to 2.9.3. The application of nanoparticles helps improve the solubility, stability, controlled release, and bioavailability of the entrapped plant extracts and helps reduce the drawbacks of non-encapsulated plant-based extracts (Vega-Vásquez, Mosier and Irudayaraj, 2020).

2.9.1 Lipid-based nanoparticles

Lipid-based nanoparticles such as nanoemulsions, liposomes, and solid lipid nanoparticles are commonly used as vehicles for antibacterial drugs (Yeh *et al.*, 2020; Lin *et al.*, 2017). Liposomes are made up of lipid bilayers made of phospholipids having enclosed aqueous compartments which can be used for the delivery of smaller molecular weight therapeutics, imaging agents, peptides, proteins, and nucleic acids (Jain and Thareja, 2019). Their sizes allow them to tolerate the release of an encapsulated drug, and prolonged exposure to the drug at the target site can enhance efficacy. Physicochemical properties such as temperature, ionic strength, and pH are very important when designing liposomes. Liposomes can fuse with mammalian cells and microbes, enabling the transport of drugs across biological membranes.

Lipid nanoparticles, such as solid lipid nanoparticles, nanostructured lipid carriers, and nanoemulsions, appear suitable as drug-carrier systems due to their very low cytotoxicity relative to polymeric nanoparticles because while positively charged at low pH to enable

RNA complexation, they become neutral at physiological pH which reduces potential toxic effects as (Kedmi *et al.,* 2010). Lipid nanocarriers are proved to have adaptable properties in terms of particle size, bilayer charge, bilayer composition, and encapsulation ability, which makes these carriers useful for drug delivery. Since lipid nanoparticles are smaller in size, this allows them to tolerate the release of an encapsulated drug, and prolonged exposure to the drug at the target site can enhance efficacy (Mukherjee, Ray, and Thakur, 2009; Pandey, 2020). While liposomes, solid lipid nanoparticles, nanostructured lipid carriers, and nanoemulsions are prone to faster degradation and can be easily engulfed by liver macrophages and destroyed, they have the benefit that they can be used for active targeting (Jain and Thareja, 2019). The structural difference between different lipid-based nanoparticles such as liposomes, solid lipid nanoparticles, nanostructured lipid carriers, and nanoemulsions is illustrated in Figure 7.

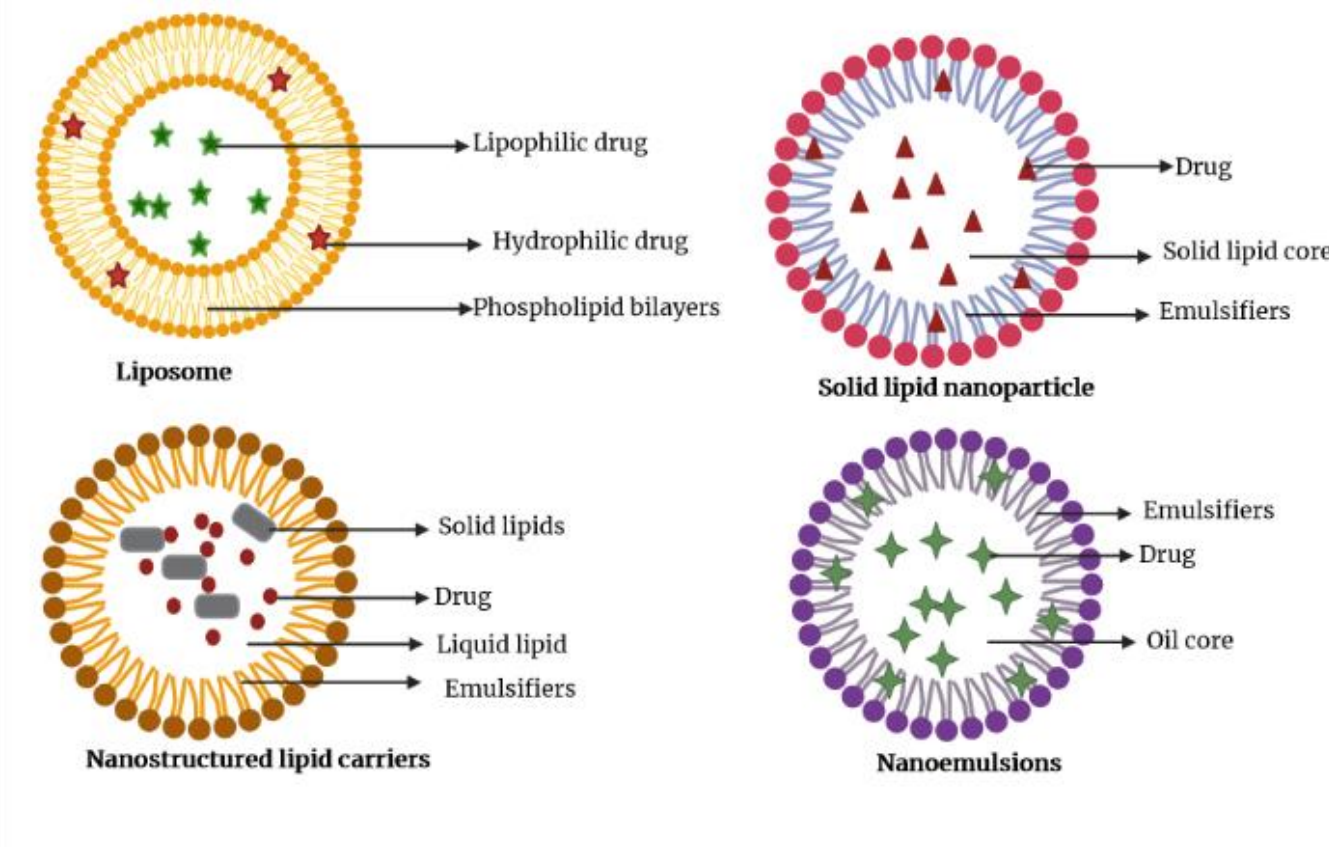

Figure 7: A schematic of different classes of lipid-based nanoparticles. Created with BioRender.com, (Shatri *et al.*, 2021).

2.9.2 Inorganic nanoparticles

Metallic nanoparticles that are made using Gold (Au), Silver (Ag), or Copper (Cu) have shown strong antimicrobial activity however, their efficacy is hindered due to reports of toxicity to mammalian cells (Sánchez-López *et al.*, 2020). Reforming the metallic nanoparticles is needed to improve biocompatibility. The unique physical and chemical characteristics of Au nanoparticles have made them candidates of choice as drug delivery carriers and bioimaging because their toxicity can be controlled by ensuring that the size of the particles remains between 4–5 nm in diameter. After all, below this size, Au

nanoparticles become catalytically active and can induce cytotoxicity (Sánchez-López *et al.,* 2020; Falagan-Lotscha, Grzincica, and Murphy, 2016). Some antibiotics, antibacterial peptides, and surfactants can be conjugated onto the nanoparticulate shell to initiate potential antibacterial activity. While Au nanoparticles have shown acceptable biocompatibility, stable storage, and easy surface functionalization in comparison to other inorganic metal nanoparticles; Ag nanoparticles have shown a broad-spectrum antibacterial to drug-resistant strains by disintegrating the bacterial wall resulting in subsequent leakage of cytoplasmic contents. (Yeh *et al.*, 2020) The superparamagnetic iron oxide nanoparticles have magnetic hyperthermia properties and are utilized as bacteria separation agents and bioimaging contrast agents for bacteria diagnosis. superparamagnetic iron oxide causes hyperthermia which causes the increased bacterial membrane permeability that kills the targeted bacteria at temperatures >45°C. Mesoporous silica nanoparticles have shown a strong antibacterial effect on bacteria with an inhibition rate of up to 30% within 3 hours *in vitro* (Tian and Liu, 2020; Mostafa *et al* 2015). The structural difference between different inorganic nanoparticles is illustrated in figure 8.

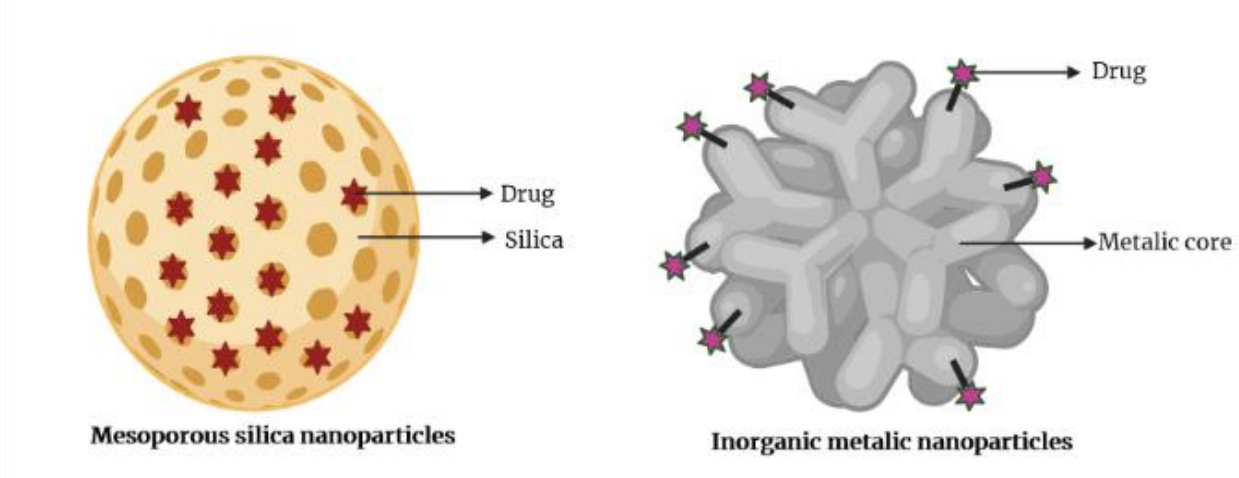

Figure 8: A schematic of structural differences of classes of inorganic nanoparticles. Created with BioRender.com, (Shatri, 2021).

2.9. 3 Polymeric nanoparticles

Polymeric nanoparticles are submicron-sized (10–1000 nm) and made up of solid polymeric carriers and they can fall into different classes as a function of their morphology, size, composition, and physicochemical properties (Gagliardi *et al.,* 2021). The therapeutic substances can either be covalently bound to a polymer backbone or physically incorporated into a polymer matrix. The biopolymers can form nanoparticles with high biocompatibility and biodegradability. Polymeric nanoparticles are classified into different categories such as polysaccharides, nucleic acids, and peptides or protein nanoparticles (Zieli´nska *et al.,* 2020). However, a study by Mukherjee *et al.,* (2009) contradicts the biocompatibility of polymeric nanoparticles. Polymeric nanoparticles such as polymersome, dendrimer, polymeric micelle, and nanosphere offer a controlled release of the nanoparticles, payload flexibility for hydrophobic and hydrophilic cargo, and easy surface modification. However, there have been some reports of particle aggregation and

toxicity (Zielińska *et al.*, 2022). Examples of polymeric nanoparticle structures are shown in Figure 9.

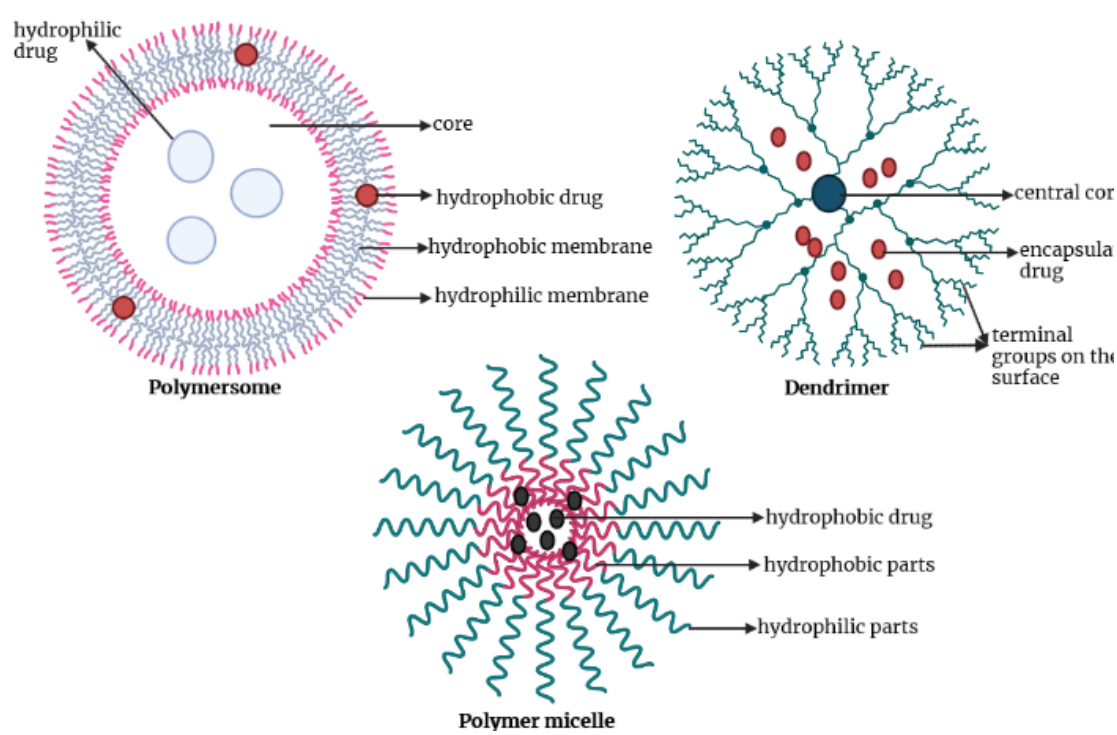

Figure 9: A schematic of structural differences of classes of polymeric nanoparticles. Created with BioRender.com, (Shatri, 2021).

Chitosan is one of the FDA-approved biopolymers with linear polysaccharides. Chitosan is composed of randomly distributed β-(1 → 4)-linked D-glucosamine and N-Acetyl-D-glucosamine and it has shown antibacterial and antibiofilm properties due to its polycationic nature's ability to disrupt bacterial membrane (Marques *et al.*, 2020). Chitosan-based nanoparticles have been broadly used as drug delivery systems. The mucoadhesive character of chitosan to gastrointestinal epithelium, and buccal mucosa is essential for the drug release of the nanoparticles as it offers a prolonged residence time in biomembranes *in vitro* and *in vivo* studies (Li *et al.*, 2018, Duan *et al.*, 2020). Solutions

of chitosan are reported to increase cellular permeability with a dose-dependent effect. This is however determined by the molecular weight and degree of deacetylation of the chitosan. The mucoadhesive properties of chitosan are caused by the positive charges on the chitosan that form interactions with the tight junction proteins occludin of the cells resulting in destabilization of the plasma membrane. Chitosan can prolong residence time in the gastrointestinal tract utilizing its mucoadhesive properties, it can also enhance drug absorption by increasing cellular permeability (Bowman and Leong, 2006). These are all major factors contributing to its widespread evaluation as a compatible polymer dutiable for use in the development of oral drugs. An *in vivo* study by Hirano, (1996) has shown chitosan to be nontoxic and biodegradable, with an oral lethal dose (LD)50 in mice of over 16 g/kg.

Although Hoshino *et al.* (2011) cautioned on the limitations of using synthetic polymers, Due to their benefiting properties such as biocompatibility and biodegradability, recent studies prove that synthetic polymers such as poly(lactide-co-glycolide) (PLGA) are generally regarded safe for use in drug delivery systems and are recognized by the United States'Food and Drug Administration (USFD) (Reddy *et al.,* 2021). PLGA nanoparticles are designed for drug delivery and aid therapeutic efficacy as they offer prolonged residence time, drug protection, and improved drug targeting abilities. Changing the ratio of lactic acid and glycolic acid can enable the control modification of the drug release and degradation rate (Essa *et al.,* 2020). Other synthetic amphiphilic polymers such as poly (malic acid) (PMLA) have also shown acceptable biocompatibility, biocompatible, and less toxic effects. The carboxyl moieties in PLMA allow the introduction of various chemical modifications such as antibodies, proteins, and specific antibiotics for

nanoparticle development (Casajus *et al.*, 2018). The United States Pediatric Formulation Initiative (US PFI) has encouraged scientists to apply new technologies such as polymeric nanoparticles as a controlled drug delivery system over a long period for pediatric drug formulations (Nieto-González *et al.*, 2021).

Coupling synthetic polymers with chitosan have several advantages such as enhanced control and target drug release, improve stability of pharmaceuticals, high and enhanced drug content when compared to other carriers, and the ability to carry both lipophilic and hydrophilic drugs, most lipids are biodegradable (Zhang *et al.*, 2021). Poly (lactic-co-glycolic acid) nanoparticles (PLGA Nanoparticles) are well recognized as an ideal drug delivery carrier for their biocompatibility and biodegradability. Burst release refers to an unpredictable and uncontrolled release of encapsulated drugs from nanocarriers when introduced to a release medium (de Azevedo *et al.*, 2017). To overcome the disadvantage of drug burst release in a gastric environment by stabilizing the formulated nanoparticles, chitosan can be used to modify the PLGA nanoparticles as shown in Figure 10 and Figure 11. The positively charged chitosan-modified PLGA nanoparticles have enhanced absorption and bioavailability due to the mucoadhesion properties of chitosan. Mucoadhesive properties of Chitosan modified PLGA nanoparticles are desirable as they can increase the residence time of drugs at the site of absorption and action which provide sustained drug release while minimizing the degradation of drugs in various body sites (Ways *et al.*, 2018).

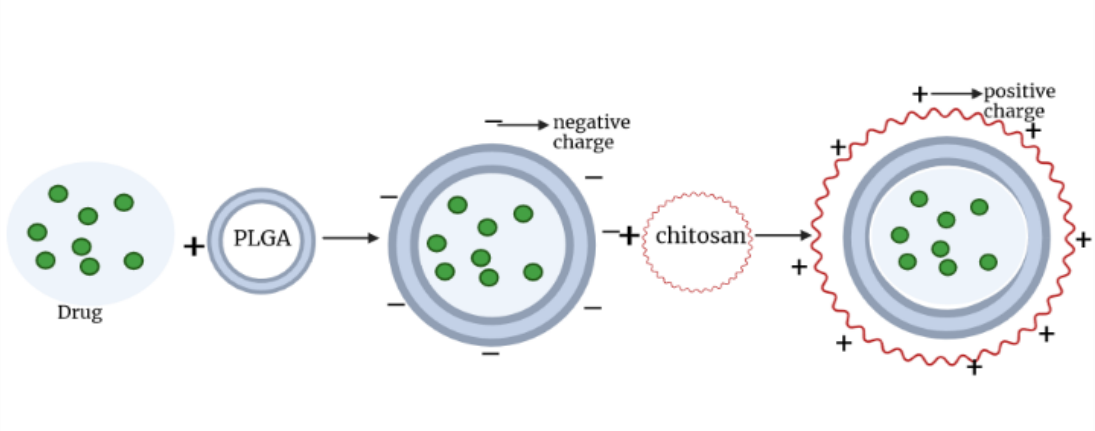

Figure 10: A Schematic diagram of the formulation of the chitosan-modified PLGA nanoparticles. Created with BioRender.com, (Shatri, 2021).

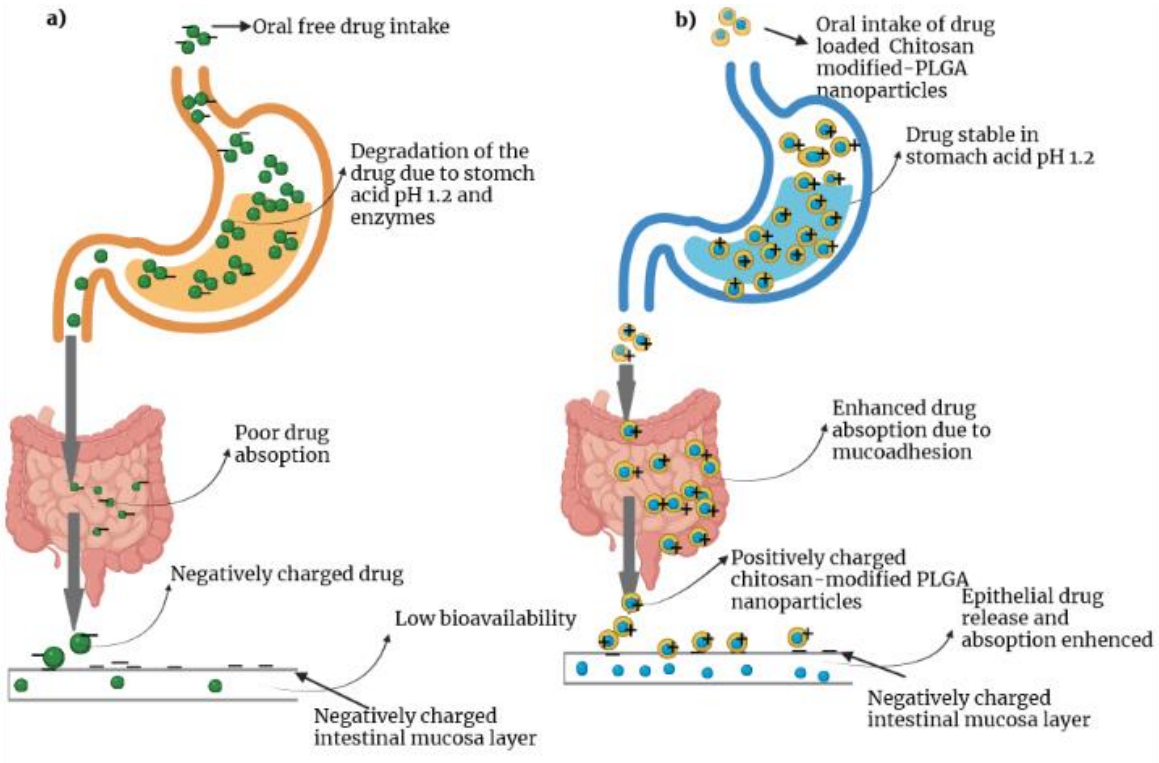

Figure 11: A Schematic diagram of the behavior of the a) free drug and b) chitosan-modified PLGA nanoparticles within the GI tract. Created with BioRender.com, (Shatri, 2021).

Alginate is another biopolymer commonly used to fabricate drug-delivery nanocarriers. Unlike chitosan, alginate is an anionic polysaccharide that is derived from the cell wall of algae (Abourehab *et al.,* 2022). Alginate-based nanoparticles have been used as carriers for compounds for treating tuberculosis and fungal infection (Hariyadi and Islam, 2020). Proteins are interesting ingredients for the preparation of antibacterial nanoparticles because of the variety of molecular weights and easy chemical modification which made proteins such as albumin and ferritin important in nano-drug delivery (Yeh *et al.,* 2020).

2.10 Food and Drug Administration-approved classes of polymeric nanoparticles that are suitable for delivering phytomedicine

The biodegradability and biocompatibility of a polymer are important properties to be monitored and evaluated continuously to maintain pharmacovigilance when designing and developing medicine. It is important to understand that good polymer biocompatibility does not ensure good biodegradability (Meganck and Baric, 2021). Among many existing polymers, only a few meet the FDA standards and are therefore approved when designing pharmaceutical products. This includes natural and synthetic polymers summarized in Figure 12 (Elmowafy, Tiboni, and Soliman, 2019).

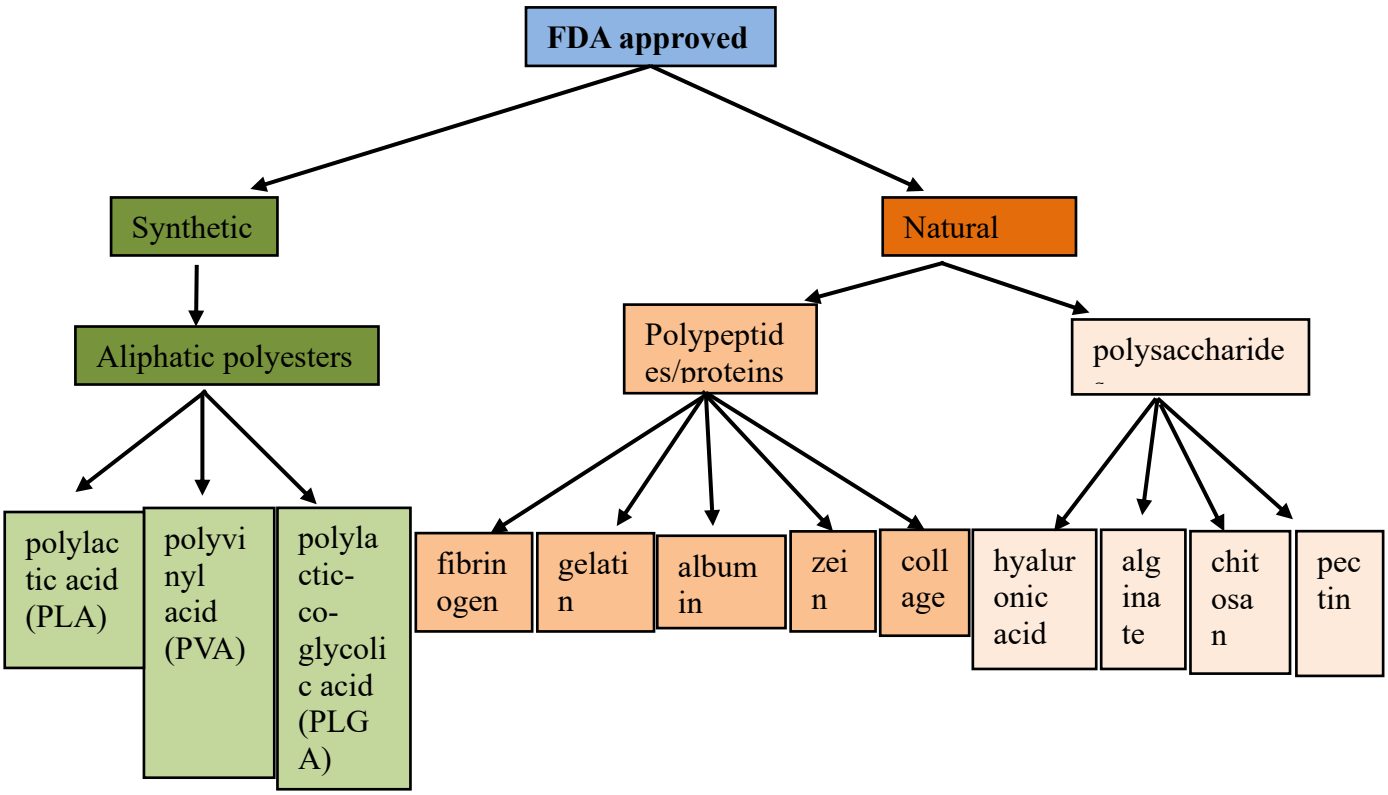

Figure 12: FDA-approved polymers for oral drug delivery

These polymers are biodegradability, biocompatibility, and non-toxic properties which makes them suitable as matrices for controlled-release drug delivery systems. PLGA has become one of the most studied diblock copolymer biomaterials for drug encapsulation and is present in several commercially available pharmaceutical products because of its slow degradation and drug release rates (Mansour *et al.*, 2010, Sung and Kim, 2020).

2.11 Polymeric nanoparticles for treating gastrointestinal tract diarrheal pathogens

Bacteria are responsible for up to 40% of diarrhoeal cases reported worldwide, thus contributing to high rates of childhood mortality in developing regions, and substantial morbidity and economic losses in developed regions (Mokomane *et al.*, 2017). Moreover, the indiscriminate use of antibiotic drugs in the last decades has triggered an increasing multiple resistance to these drugs, which represents a serious global socioeconomic and public health risk (Cano *et al.,* 2020). *Salmonella* species, *Vibrio cholera*, *Campylobacter jejunum,* and *Helicobacter pylori* are considered among the highest causative agents of gastrointestinal conditions. Gastroenteritis has become a major public health problem worldwide thus efforts in identifying an alternative therapeutic approach against *Salmonella* and other diarrheal pathogens are gaining much interest. Table 2 summarizes the recent preclinical novel drug-loaded polymeric nanoparticles for the treatment of *Salmonella, Campylobacter, V. cholera,* and *E. coli* (Cano *et al.,* 2020; Spirescu *et al.,* 2021).

Table 2: Recent preclinical novel drug-loaded polymeric nanoparticles for the treatment of *Salmonella, Campylobacter, V. cholera,* and *E. coli*

Bacteria	Loaded molecule	Polymeric matrix	Surface modification	References
S. Typhimurium	Cryptdin	Chitosan	None	Cano *et al.,* 2020; Spirescu *et al.,* 2021,
V. cholera	Unknown	PLGA	Monosialotetrahexosylganglioside	Spirescu *et al.,* 2021, Cano *et al.,* 2020
C. jejunum	CpG oligodeoxynucleotide	PLGA	None	Cano *et al.,* 2020
E. coli	Gentamycin	Chitosan	none (pH-responsive)	Spirescu *et al.,* 2021

2.12 Bioactivity of nanoparticles as carriers for plant-based pharmaceutical products

It is important to retain the strength and effectiveness of a drug to be encapsulated in the nanocarriers (Pereira *et al.,* 2018; Crucho and Barros, 2017). Plants are the natural source of phytochemical compounds such as phenolic acids, anthocyanins, flavonols, coumarins, alkaloids, tannins, saponins, and flavan-3-ols have gained great interest due to their functional properties, such as antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial effects (Pereira *et al.,* 2018). However, their application has been limited since their stability can be affected by factors such as storage period, temperature, light, pH, oxygen, and interaction substances (Andrés-Bello *et al.,* 2013). During digestion pH, enzymes, interaction with other nutrients, and acid can affect the effectiveness of bioactive compounds (Rein *et al.,* 2013). Encapsulation of these plant extracts rich in phytochemical compounds with FDA-approved polymers could address some of these

problems such as bacterial infection and enhance their functional properties and this is one of this study's hypotheses (Zhu *et al.,* 2014).

2. 13 Physicochemical properties to consider when formulating polymeric nanoparticles

The main components to consider when designing nanoparticles as a delivery system are managing particle size, and surface properties as well as the release of drugs to fulfill the specific need (Rizvi and Saleh, 2018). These characterizations are very critical to controlling nanoparticles' performance *in vitro* as well as *in vivo*. Electron microscopy is used to determine the surface morphology and size of the nanoparticles. Dynamic light scattering (DLS) and photon-correlation spectroscopy are used to measure the particle size and size distribution (Jain and Thareja, 2019). These properties affect the release profile of encapsulated agents. Smaller nanoparticles result in a large surface area which increases the entrapment and fast release of the drug with slow drug diffuse out from the inner layer of the nanoparticles. Although smaller nanoparticles come with several advantages, smaller nanoparticles tend to aggregate while delivery and storage (Singh and Lillard, 2009).

2.13.1 Dynamic light scattering and nanoparticle size analysis

Dynamic light scattering (DLS) is commonly used to compute nanoparticle size in a suspension utilizing photon correlation spectroscopy (PCS). DLS can analyze nanoparticles of sizes between 1 and 1500 nm. DLS instrument detects scattered beams of laser light with a photon detector and the intensity of the scattered light is proportional to the size of the nanoparticles which are monitored (Stetefeld, McKenna, and Petel, 2016).

2.13.2 Microscopic techniques used in morphological analysis of Nanoparticles for targeted drug delivery

Scanning and Transmission electron microscopy is used to determine the surface morphology and size of the nanoparticle. Unlike light scattering-based techniques, with microscopy, individual particles are measured by the Coulter Counter and microscope techniques hence, these techniques provide the advantage of direct close measurement. Scanning Electron Microscopy allows the analysis of dry powdered nanoparticles. Transmission Electron Microscopy is used to analyze the morphology of nanoparticles within a suspension (Liu, 2021; Asadabad and Eskandari, 2015).

2.13.3 Zeta potential and its importance in nanoparticle stability

The Zeta potential of the nanoparticles refers to the surface charge of nanoparticles in solution (Clogston, and Patri, 2011). The surface charge of the nanoparticles plays a significant role in the physical stability and dispersibility of the nanoparticles' dispersion as well as the *in vivo* performance. The determination of surface charge sanctions forecasts the long-term storage stability of nanoparticles (Zielińska *et al.,* 2020). Higher zeta potential may result in particle aggregation because of electric repulsion between particles. Determination of the zeta potential gives an idea about the net charge of nanoparticles hence, providing a sign of the electrical repulsion or attraction among the particles as per their charge in a liquid suspension. A zeta potential greater than ±30 mV is required to stabilize the nanoparticle's dispersion by blocking the contact between the nanoparticles because of electric repulsion. The surface electrical potential of nanoparticles is vital in nanoparticle formulation because it modifies the interaction with adjacent nanoparticles and the biological system (Soares *et al.,* 2020).

2.13.4 Drug release and cellular uptake of nanoparticles

An important aspect of nanoparticles as a carrier for drug delivery is to understand the way and level to which the therapeutics are released. Release profile studies are performed in quite an analogous manner to encapsulation efficiency determination assays which are measured for a time interval to find out the release mechanism. This is done by monitoring the concentration of the nanoparticles within a solution. To attain efficient disease management, the concentration of released therapeutic agents from polymeric matrices should be within the therapeutic window with minimal fluctuation should be over a prolonged period at the intended site of action (Mansour *et al.,* 2010). The release of a therapeutic agent can be controlled by diffusion, erosion, osmotic-mediated events, or combinations of these mechanisms. In addition to the physical and chemical characterization of nanoparticles, the biological responses of nanoparticles can be determined in animal cell models before introducing the nanoparticles *in vivo* (Patra *et al.,* 2018). Nanoparticles uptake study can be evaluated using monolayer cell culture models and this offers useful information about the uptake of nanoparticles by cells, the therapeutic potential of the drug, and the side effects of the carriers. Flow cytometry and confocal microscopy are used to analyze cells for cell uptake mechanisms (Behzadi *et al.,* 2017). A fluorescent marker is a useful tool and can be conjugated with the drug and allow monitoring of the drug release both *in vivo* and *in vivo*. However, when this is not done correctly, unbound fluorescent dyes can change the chemical and physical properties of the nanoparticles, and this led to wrong conclusions (Kokot *et al.,* 2021). Physicochemical characteristics of nanoparticles determine the mechanism of particle translocation such as

phagocytosis, macropinocytosis, and clathrin- or caveolae-mediated endocytosis (Foroozandeh and Aziz, 2018).

Is well established from this review that the burden of gastroenteritis and antibiotic resistance are major global health burdens. The review also highlighted that *Salmonella* and *E. coli* are rapidly developing resistance to commercially available medicine. However, for most African countries including Namibia, there is limited data to show resistance patterns to clinically used antibiotics. Various treatments available for gastroenteritis also have numerous side effects and this calls for alternative treatment methods to combat the issue of resistant pathogens. While medicinal plants are an alternative that has been used traditionally for centuries due to their bioactive compounds with potential antibacterial and antidiarrheal properties, they too have limitations relating to their release properties and the challenges they face within the GI tracts including acid and enzymatic degradation and poor solubility and. However, in Namibia, this knowledge is not well documented and can be lost over time. Nanosystems among other delivery systems can help improve the effectiveness of medicinal plants as an alternative treatment for gastroenteritis and eliminate some of the challenges faced by medicinal plants within the GI tract. Hence, in this study, Knowledge of the use of medicinal plants in northern Namibia will be documented, validated and the lead plants with potent antibacterial activity will be encapsulated into chitosan-modified poly (lactic-co-glycolic acid) nanoparticles and tested against clinical isolates of *Salmonella* and Enteropathogenic *E. coli.*

2. 14 References

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CHAPTER 3: ETHNOMEDICINAL USE AND PHYTOCHEMICAL ANALYSIS OF MEDICINAL PLANTS USED TO TREAT GASTROINTESTINAL CONDITIONS BY AWAMBO PEOPLE IN IIKOKOLA VILLAGE, NAMIBIA

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3.1 Abstract

Namibia is ethnically diverse and rich in indigenous knowledge of the medicinal uses of plants. However, this knowledge in treating gastrointestinal conditions is not well documented despite its contributions to the healthcare system in Namibia. This study aimed at documenting the ethnomedicinal uses of plants used to treat gastrointestinal conditions in the Iikokola village and validating their use through phytochemical analysis. A survey was conducted using semi-structured questionnaires with consenting knowledge holders. Voucher specimens were collected and deposited with the National Herbarium

of Namibia for botanical nomenclature. Among the 26 knowledge holders approached 23 participated in the study, of which 16 were females and 7 were males. A total of 19 plant species belonging to 10 plant families were reported to treat at least one gastrointestinal condition and 14 other ailments. Most species belong to Fabaceae and Combretaceae families (Family Importance Value=100). The gastrointestinal use of 32% of the plants documented in this study has not been reported elsewhere. Diarrhea, vomiting, and stomach pain are frequently managed using medicinal plants. Decoctions from roots of *Grewia tenax*, *Terminalia sericea*, *Corchorus tridens*, *Albizia anthelmintica,* and *Lantana camara*, were mostly used for gastrointestinal ailments. Oral administration was the only route used in the administration of gastrointestinal remedies, among all age groups. Phytochemical compounds such as coumarins, flavonoids, saponins, and tannins which have been linked to the antidiarrheal activity of plants, were detected in organic and aqueous extracts. Organic extracts of *T. sericea* and *G. tenax* showed higher total phenolic and flavonoid contents of ≤ 169.46 and ≤ 74.03 mg QE/g respectively. These phytochemical properties add value to their ethnomedicinal use is rational although antimicrobial properties and safety profiles should be determined. The traditional uses of medicinal plants reported in this study will help safeguard and guide future research aiming at developing plant-based treatments for gastrointestinal conditions.

Keywords: Ethnomedicine; Gastrointestinal conditions; Decoction; Oral administration; Photochemistry

3.2 Introduction

People in rural communities in developing countries rely on medicinal plants as a primary health option for treating different illnesses (Vasanthi, ShriShriMal, and Das, 2012). The knowledge on the use of plants is passed on orally from generation to generation, and only a little of this knowledge is documented (Meragiaw, Asfaw, and Argaw, 2016). In both rural and urban settings, communities sometimes opt to use medicinal plants because they are cost-effective and readily available (Randrianarivony *et al.,* 2017). Rural communities believe in the effectiveness of traditional treatment methods and specifically in the uses of the plant, hence they prefer the use of medicinal plants over allopathic medicine. Research conducted towards drug discovery, documentation, and validation of the uses of medicinal plants has increased tremendously due to an increase in antibiotic resistance, and the reemergence of infectious diseases globally (Prestinaci, Pezzotti, and Pantosti, 2015). Plants have been reported to be sources of several modern drugs and supplements used to treat different ailments worldwide (Koparde, Doijad, and Magdum, 2019).

Since medicinal plants have a long history of use and are readily available for use in most developing countries; they can be good sources of new medicines for treating communicable and non-communicable diseases (Randrianarivony *et al.,* 2017). Many plants are categorized as supplements that can be used for general body health and this limit detailed research on their safety and efficacy (Sahoo, 2018). The World Health Organization (WHO) recommends documenting the use of medicinal plants by indigenous people from different parts of the world and Namibia is one of the 179 countries that have contributed to the WHO's report on traditional and complementary medicine (WHO, 2019). Although many plants are edible and have been shown to have

medicinal value in preventing and alleviating numerous ailments; there is limited information on their long-term uses and possible side effects that could occur when they interact with other medicine (Mutua *et al.*, 2016). Studies conducted on the pharmacological properties of plants have also shown that different plants are enriched with secondary metabolites that enable them to fight infections and alleviate different ailments when consumed by humans (Kennedy and Wightman, 2011; Iikasha, Bock, and Mumbengegwi, 2017).

In Namibia, the use of ethnomedicinal plants is practiced but limited studies are documenting and validating the uses of medicinal plants indigenous to Namibia (Chinsembu, Hijarunguru, and Mbangu, 2015, Shatri and Mumbengegwi, 2020; du Preez, Shingenge and Mumbengegwi, 2020). Namibia is divided into 14 administrative regions which are further subdivided into 121 constituencies. Omusati region is in North-central Namibia, and it has 12 constituencies or the 13 tribes in Namibia (Figure 1) (Haindongo, 2022), the "Aawambo" tribe predominates the Omusati region (Shusko, 2015) and they have used ethnomedicinal plants as a source of primary health care (Shatri and Mumbegegwi, 2021). Omusati region's population of 240 900 is the third-largest population in Namibia, preceded by those of Khomas and Ohangwena (341 000 and 242 700 respectively (Mounton, 2014). Omusati is divided into 12 constituencies namely, Anamlenge, Elimi, Etayi, Ogongo, Okalongo, Okahao, Onesi, Oshikuku, Otamanzi, Ruacana, Outapi, and Tsandi. The Iikokola village is located in the Tsandi constituency which holds a population size of 13 495. Most people of the Omusati region (94%), are Oshiwambo-speaking (Steytler, 2014). The Omusati region is reported to be one of the poorest regions with poor sanitation and higher reports of diarrhea in Namibia (Iikasha et

al., 2019; Mouton, 2014). Moreover, the Iikokola village is located far from referral hospitals which makes the use of medicinal plants crucial as the first line of treatment for people in this community. This may be the reason why its population relies on ethnomedicine as a health care option for treating different ailments. While Lumpkin (1994) has shared insight into the traditional healing practices of people in the Omusati region, his study did not provide details, especially for the treatment of gastrointestinal conditions using medicinal plants that are addressed in this study. Another study conducted by Shatri and Mumbegegwi (2020) in the Northern part of Namibia focused on the antibacterial activity of the leaf extracts of the ethnomedicinal plants *Terminalia sericea, Boscia albitrunca,* and *Sesamum capense* from the Kunene Region of Namibia as a treatment for common causes of bacterial diarrhea, such as *Shigella boydii*, *Salmonella typhi*, and *Escherichia coli*. While that study has quantified the phytochemical and antibacterial activity of *T. sericea, B. albitrunca,* and *S. capense* of the plants that are also reported in this study, a full description of the parts used, methods of preparing medicine, route of administration, and recovery period are not provided in that study. Despite Namibia's diversity of vegetation used for medicinal purposes, there are limited studies that validate the uses of medicinal plants indigenous to Namibia. This limits the mainstream use of medicinal plants in treating gastrointestinal conditions and other disorders in the Namibian population. Moreover, the herbal medicine system is still largely informal, and not possible to determine the veracity of many claims yet there is a reliance on herbal medicine.

Phytotherapy is a significant therapeutic approach in treating functional gastrointestinal diseases with approximately 34% of plant species documented by Kelber, Bauer, and

Kubelka., (2017). This is because plants can produce many bioactive compounds such as alkaloids, flavonoids, coumarins, anthraquinones, steroids, tannins, and vitamins that may complement the needs of the human body by acting as natural antioxidants (Altemimi *et al.*, 2017). When a plant is analyzed for phytochemical compounds based on its traditional uses then it is needed to prepare the extract as described by the traditional healer to mimic as closely as possible the traditional 'herbal' drug. Based on other studies, organic extracts prepared using methanol, ethanol, and ethyl-acetate are reported to contain higher quantities of phytochemical compounds with potent antibacterial and antidiarrheal properties *in vitro* and *in vivo* (Alam *et al.*, 2021; Sasidharan *et al.,* 2011), hence in this study, water that is used in the traditional setting as per the results of this study was used to prepare aqueous extracts, while methanol was used to prepare organic extracts to provide a point for comparing the quantities of phytochemical compounds present in these extracts. Moreover, the selection of solvent system largely depends on the specific nature of the bioactive compound being targeted with methanol, ethanol, and ethyl-acetate being the preferred organic solvents for extracting polar compounds (Sasidharan *et al.,* 2011). Moreover, several studies have shown that plant remedies can kill diarrheal pathogens due to the presence of phytochemical compounds such as alkaloids, tannins, flavonoids, coumarins, terpenoids glycosides, phytosterols, and saponins (Tadesse *et al.,* 2017; Iikasha *et al.,* 2017; Derebe *et al.,* 2018). The present study aimed to document the ethnomedicinal uses and quantify the contents of phenolic and flavonoids in selected plants used to treat gastrointestinal conditions and other ailments in the Iikokola village, Omusati region. Phenolic and flavonoids were selected because they are reported to be responsible for the antioxidant properties op plants which have various biological

properties. Polyphenols such as flavonoids are specifically reported to have broad-spectrum antibacterial (against both gram-positive and gram-negative bacteria), and antifungal properties as well as anti-inflammatory properties. Hence, they were analyzed in further detail compared to other compounds. Manso et al., 2021). This is important to the Namibian population currently using these plants as complementary and alternative medicine as it adds value regarding phytoconstituents that are present in the plants while preserving the knowledge and providing bases for the knowledge for future generations aiming at developing next-generation plant-based medicine.

3.3 Material and methods

3.3.1 Study area and rationale for choosing the study site

This study was carried out in Iikokola village, Uukwaludhi district in the Omusati region, Namibia (Figure 1). The selection of the Iikokola village was based on the fact that there is no documented knowledge of the ethnomedicinal uses of plants by the Aawambo people of Iikokola village. Moreover, since the village is located about 20 km from the referral hospital ethnomedicine is frequently used as a first and alternative treatment method in this village, moreover, participants perceived the willingness of knowledge holders in the communities to cooperate with the objectives of the study.

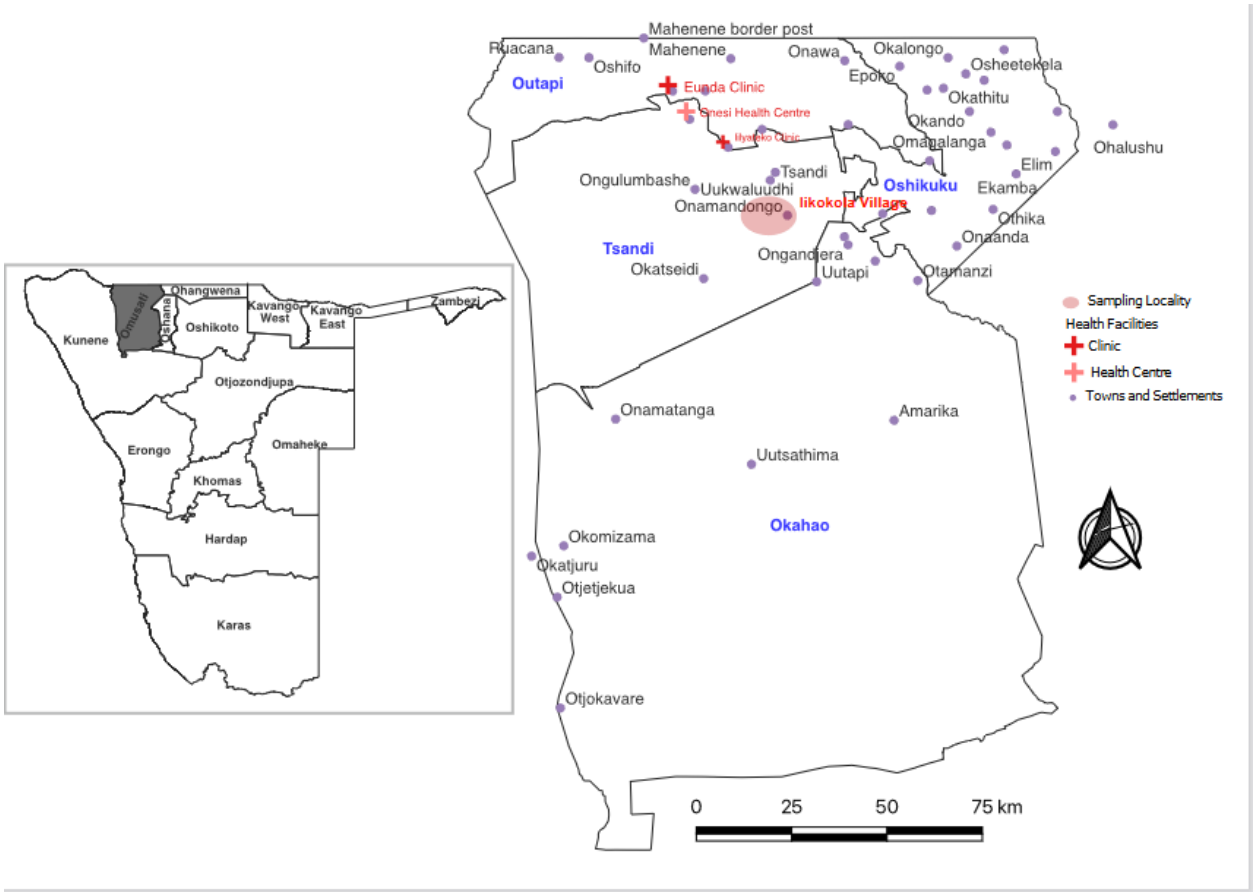

Figure 1. A Regional map of Namibia highlighting the study area for this study "Iikokoka village. Created by Haindongo, in 2022 in QGIS 3.24.

3.3.2 Ethnobotanical field survey and data collection

This study was carried out between April 2017 and October 2021. A plant collection permit (2221/2017) in Appendix B was obtained from the National Commission on Research Science and Technology (NCRST) and an ethical clearance (Appendix A) to conduct this study was obtained from the University of Namibia. Semi-structured interviews were conducted with participants in this study. The interviews were conducted face-to-face with participants who were ≥18 years old from the community. The Snowball technique was used in the sampling of participants, whereby participants identified others as referrals for participation in the study. A total of 26 knowledge holders from the study

area were approached based on a referral from the village headman. The design of the questionnaires and levels of dissemination of data obtained in this study was guided by the policies on the governance of indigenous natural products in Namibia (Ndeinoma, Wiersum, and Art, 2018). The purpose of the study, methods of data collection, and intention to document data were thoroughly explained to knowledge holders that were approached in the indigenous language (Oshiwambo). Subsequently, detailed interviews for purposes of data collection were carried out with only knowledge holders that agreed to participate in the study and who signed an individual written prior informed consent form (Appendix D). Data was collected from 23 knowledge holders through interviews using a semi-structured questionnaire with predetermined open-ended and direct questions (Appendix E). The questionnaire included the local plant name, source of knowledge about plants, plant parts used, methods of harvesting, times of harvesting, preservation technique, diseases and ailments treated, routes of administration of the herbal remedies' modes of preparation, and time to recovery from ailment post-treatment (Appendix E). Demographic information of the informant such as age, gender, and educational background was also collected. Knowledge holders were interviewed individually, and the interviews were conducted in their homes and places where they collected plants for treatments. Interviews were conducted in Oshiwambo and translated into English.

3.3.3 Plant specimen collection, preservation, and identification

Plant species used to treat gastrointestinal conditions were collected with the help of the knowledge holders interviewed in places where they normally collected plant materials for their use. For each plant, a voucher specimen was collected, pressed, and sent to the

National Herbarium of Namibia for scientific identification together with a plant collection form (Appendix F and G). Family names of identified plants were verified using a plant atlas of Namibia. Plant parts of different frequently used plants were collected for phytochemical screening and other biological assays. The collected plant materials were washed with distilled water to remove sand and stored and stored in the shade for 4 weeks to dry. After drying, plant materials were crushed into powder using an industrial blender.

3.3.4 Quantitative analysis of the ethnomedicinal information

The knowledge of the medicinal plant uses was assessed quantitatively using the relative frequency of citation (RFC), family importance value (FIV), and use value (Uv). The RFC and FVI were calculated to quantitatively determine the consensus between informants on the use of the plant in the community as it gives the local importance of the species and the plant family. The RFC was calculated using the standard method as described by Napagoda *et al.* (2018).

RFC=FC/N

RFC should be (0<RFC<1)

The RFC value for species and families of medicinal plants is based on the citing percentage of an informant for a particular species or plant family. FC is the number of informants who use the species traditionally, while N is the total number of informants who participated in the study.

The family importance value (FIV) was calculated by taking the percentage of informants mentioning the family (Napagoda *et al.*, 2018):

$$\text{FIV} = \frac{\text{FC(family)}}{\text{N}} \text{X } 100$$

Where FC is the number of informants mentioning the family, while N is the total number of informants participating in the study.

3.3.5 Plant material

The five plants with higher RFC values such as *T. sericea* (twigs and seeds), *L. camara* (twigs and seeds), *G. tenax* (roots and twigs), and *A. anthelmintica* (twigs and roots) were subjected to further phytochemical screening. Plant materials were selected for this study based on the utilization of ethnomedicine by knowledge holders in Iikokola village to manage different gastrointestinal conditions. Plant materials were collected for extraction and further quantified for phytochemical compounds that are linked to the antidiarrheal properties of medicinal plants.

3.3.6 Preparation of crude extract

Plant material prepared as described namely *T. sericea* (twigs and seeds), *L. camara* (twigs and seeds), *C. tridens* (Whole plant), *G. tenax* (roots and twigs), and *A. anthelmintica* (roots and twigs) were ground to a fine powder using an industrial grinder. In this study, water that is used in the traditional setting as per the results of this study was used to prepare aqueous extracts, while methanol was used to prepare organic extracts to

provide a point for comparing the quantities of phytochemical compounds present in these extracts. For organic extracts, 20 grams of the plant materials were added to 100 ml methanol and mixed in a shaker for 48 hours at room temperature. While aqueous extracts were prepared by adding 20 grams of the plant materials to 100 ml distilled water and keeping the mixture in a water bath with boiling water for 3 hours. After filtration through Whatman no 1 filter papers, the filtrates were concentrated by rotary evaporation at 40 °C and then freeze-dried to form a powder. The powdered extracts were stored at -20ºC. The percentage yield was calculated using the formula:

% yield = (Mass of plant extract/Mass of plant material) x 100.

3.3.7 Screening for phytochemical compounds of selected plant extracts

Qualitative detection of aqueous decoctions and methanol extraction mimicking the maceration technique of *T. sericea* (twigs and seeds) *L. camara* (twigs and seeds), *G. tenax* (roots and twigs), *Corchorus tridens* (whole plant), and *A. anthelmintica* (roots and twigs) were used to screen for the presence of flavonoids, anthraquinones, alkaloids, tannins; steroids and coumarin by Thin Layer Chromatography (TLC). The analysis was performed using solvent systems, chromogenic reagents, and positive controls as described by Harborne (1998) with minor modifications. Modifications included the difference in the volume of the solvent system added to the tank and the amount of extract loaded on the TLC plate. Details of chromogenic reagents, mobile solvents, and positive controls used for the TLC method are provided in Appendix K. Spots of different compounds were observed under UV light at 360nm and the retention factor (Rf) value

of different spots was calculated using the formula: Rf= Distance traveled by compound divided by Distance traveled by solvent. These values were then recorded.

3.3.8 Total phenol content analysis

Total phenolic content was analyzed by spectrophotometry using a modified Folin-CIO calteu colorimetric method. For each extract, 4 mg was dissolved in 1 ml of methanol (Dinakaran, Chelle, Avasarala, 2019). The mixtures were vortexed for 5 minutes and left to stand at -20 °C for 24 hours. The extracts were centrifuged at 1 xg for 5 minutes and 1 ml of the filtrate was added to a falcon tube containing 1.5 ml of distilled water. To this mixture, 0.125 ml of Folin-CIO calteu reagent was added. The mixtures were allowed to stand for 6 minutes at room temperature. 1.25 ml of 7% sodium carbonate and 3 ml of distilled water were added, and the solutions were allowed to stand for 90 minutes at room temperature to allow color formation. The absorbance was read at 550nm using UV-Spectrophotometer. Gallic acid was used to prepare a standard curve (1-10 µg/ml; $y=16.616+ 0.4029$, $R^2=0.999$; y is the absorbance, x is the solution concentration) as shown in the supplemental data Appendix H.

3.3.9 Total flavonoids content analysis

Total flavonoid contents of aqueous and organic extracts were determined according to the modified described method using quercetin as a reference compound. For each extract, 4 mg was dissolved in 1 ml of methanol (Dinakaran *et al.,* 2019). The mixtures were vortexed for 5 minutes and left to stand at -20 °C for 24 hours. The extracts were centrifuged at 100 rpm for 5 minutes and 0.5 ml of the filtrates were added to a falcon tube containing 0.5 ml of Aluminium trichloride (500 µg/ml). The mixtures were diluted

with 12.5 ml of methanol and allowed to stand at room temperature for 40 minutes. The absorbance was read using a UV-visible spectrophotometer at 400 nm. Different concentrations (0.01- 0.1 mg/ml) of quercetin were prepared and the total flavonoid content was determined using the same method described using the linear graph in Appendix H of the supplementary data. All experiments for determining the total flavonoid and phenol contents were repeated three times. The values are expressed as mean± standard deviation.

3.4 Results and Discussion

3.4.1 Demography of informants

Among the 26 knowledge holders approached, 23 agreed to participate in the study, of which 16 were females and 7 were males. All participants reported that they acquired knowledge on the use of plants from parents (71.43%) and grandparents (28.57%) confirming that this knowledge was shared orally down the generations. However, 57.12% of the knowledge holders reported that there is a lack of interest in the youth within their households in learning traditional healing methods (Table 1) meaning this knowledge is now being lost as it is no longer being shared as was done with previous generations.

According to the World Health Organization, ethnomedicine is a "Total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment or physical and mental illness" (World Health Organization, 2002). Interviewed medicinal knowledge holders of the Iikokola village mostly relied on plant-based remedies for treating common gastrointestinal, and

other disorders such as respiratory disorders, musculoskeletal disorders, cardiovascular disorders, and integumentary disorders are also managed using the same plants. This finding is consistent with that of Adhikari *et al.* (2019), on the broad spectral use of plants to treat different ailments. While poor sanitary conditions and poor dietary habits could be the causes of the reported gastrointestinal cases in Iikokola village since the Omusati region is one of the poor regions in Namibia according to Mouton (2014). Other factors such as the fact that the village is located about 20 km from the referral hospital, could explain why community members make use of plants to manage different ailments.

Most knowledge holders were women and women were heads of most houses in Iikokola village and were the primary caregivers. This agrees with Mashile, Tshisikhawa, and Masevhe, (2018) from Mpumalanga province, South Africa, where the adult females were well-informed on the uses of plants compared to males. Medicinal plant indigenous knowledge has been passed on orally from generation to generation among members of the same ethnic groups. In this study, some of the interviewed youths have shown interest in the use of plants as an alternative method of treatment although most elders interviewed reported a lack of interest in the youths of their households. Other studies have reported that urbanization and lack of oral transmission of knowledge from generation to generation have resulted in a loss of valuable knowledge and a lack of interest among youths in learning the ethnomedicinal uses of plants to alleviate different ailments. This is of great concern since most of this knowledge is found among elders and can easily be lost with time if not documented. (Raj *et al.,* 2018; d'Avigdor *et al.,* 2014). Studies of this kind are relevant at a national level as they ensure that ethnomedicinal knowledge for each community is documented and preserved for future generations (Boadu and Asase, 2017).

This will help guide future research aiming at developing treatments for both communicable and non-communicable diseases. This will also help preserve ethnic heritage for future generations.

Table 1: Statistics on the usage of medicinal plants for the treatment of gastrointestinal ailments

Parameter	Percentage (%)
1. Demographic data of regular users	
1.1 Gender	
Male	30.0
Female	70.0
1.2 Age group (years)	
20-45	43.48
46-60	17.39
61-80	43.48
1.3 Educational background	
University degree	43.48
Ordinary level of education	13.04
No education	43.48
2. Source of knowledge	
2.1 From family members	71.43
2.2 Neighbors/ friends	28.57
2.3 Doctors/traditional healers	0
2.4 Media	0
2.5 Own experience	0
3. Educating children on the ethnomedicinal knowledge	
3.1 Yes	42.86
3.2 No	57.12
4. Influence of social media on traditional healing	
4.1 Positive influence	60
4.2. Negative influence	40

5. Reason for using plants	
5.1 Safe /fewer side effects	71.43
5.2 Previous success	28.57
5.3 Easy access to plant materials	42.86
5.4 Higher cost western medicine	0
5.5 Lack of modern medicine	42.86
5.6 Failure of western medicine	71.43

3.4.2 Documentation of ethnomedicinal uses of plants

Although western medicine has taken over the lead from herbal medicines in the treatment of human diseases, the use of medicinal plants has increased in recent years globally, as they are believed to be safer than modern medicines with few or no side effects, and they are readily available to everyone (Nagalingam, 2017). A total, of 19 plant species were documented in this study however only 13 plants belonging to 10 plant families were authenticated since there were no voucher specimens for the other 6 plants (Table 2) by their local and botanical names. Of the 10 plant families, members of the Fabaceae, Combretaceae, and Malvaceae were most used (FIV of 100, 100, and 86.96 respectively) by the knowledge holders (Table 2.). The life form of most plants is either trees (46 %) or shrubs (46 %) (Figure 2). All plants documented in this study are used to treat at least 1 gastrointestinal condition such as diarrhea, stomach pain, bloating, stomach discomfort, vomiting, stomach worms, indigestion, and rectal bleeding. A total of 14 other human ailments are also managed using the documented plants (Table 3). Based on the participant's information, 71.43% prefer using herbal medicine because it has fewer side effects compared to modern medicine. Furthermore, 42.86 % of participants reported that plants are easily accessible, and in most cases, they lack access to western medicine hence they use medicinal plants to manage diarrhea, indigestion, vomiting, and stomach pain

during the night, especially with children. Moreover, 71.43 % of the participants reported that, in cases such as treating rectal bleeding, they prefer using herbal medicine due to the high effectiveness and quick relief in comparison to western medicine (Table 3). Participants however reported that they do not normally make use of western and traditional medicine simultaneously as this may cause death.

Table 2: Family importance value of medicinal plants from Iikokola village, Omusati region

Family name	**FIV**
Malvaceae	85.71
Combretaceae	100
Lamiaceae	28.57
Moringaceae	28.57
Rhamnaceae	42.86
Caryophyllaceae	42.86
Fabaceae	100
Capparaceae	28.57
Pedaliaceae	57.14
Verbenaceae	71.47

In Namibia, there are limited reports on the ethnomedicinal uses of plants to treat and manage diarrheal diseases. Within the 14 regions in Namibia, currently, only knowledge from certain regions such as Oshikoto and Zambezi has been published (Chinsembu, 2016; Cheikhyoussef *et al.,* 2011). Studies conducted by Chinsembu, (2016) and Cheikhyoussef *et al.* (2011) on the ethnomedicinal uses of plants in Zambezi and Oshikoto

respectively have also identified plants such as *T. sericea*, and *A. anthelmintica*, to be among the commonly used plants in these regions for treating diarrhea. *T. sericea* extracts are reported to have antibacterial activity against gastrointestinal pathogens such as *S. marcescens and H. pylori* (Mapiye, 2019). Based on this study, roots are commonly used in preparing medicine. However, twigs and leaves are also used to preserve the plants.

The ethnomedicinal uses of most of the plants identified in this study are not documented in Namibia, hence the findings of this study are the first of its kind, especially on the ethnomedicinal uses of plants such as *L. camara, S. capense, O. kilimands, P. camestris, O. kilimands, C. tridens,* and *G. tenax* to treat gastrointestinal conditions. Similar studies on the ethnomedicinal uses of plants have however been reported in other parts of the world and have coincided with the findings of this study on the ethnomedicinal uses of these plants have been reported in other parts of the world. However, preparation methods, recovery periods, and parts used differed with each ethnic group (Mongalo *et al.,* 2016; Kigen *et al.,* 2014; Mohamed *et al.,* 2013).

Table 3: Ethnomedicinal uses of plants used to treat diarrhea and other ailments in Iikokola village

Voucher specimen no.	Botanical name	Family	Local name (Oshiwambo)	Habit	Part used	Gastrointestinal uses	Preparation Method	Administration Route	Recovery Period	RFC	Other uses
BRL 30	*Corchorus tridens L.*	Malvaceae	*Okaliipute*	Climber	Whole plant	stomach pain, diarrhea	Infusion	Oral Topical	1 day 4 days	0.71	wound healing
BRL 33	*Grewia tenax (Forsak.)Flori*	Malvaceae	Oshishegele	Shrub	Roots Twigs	Diarrhea	Decoction	Oral	1-3 days	0.71	Cough, Meningitis
BRL 35	*Terminalia prunioides. M.A. Lawson*	Combretaceae	Ohama	Tree	Roots Twigs	diarrhea, stomach pain	Decoction	Oral	1 hour- 7 days	0.17	abdominal pain, cough, sore throat
BRL 38	*Ocimum kilimandscharicum*	Lamiaceae	Onjilikau	Shrub	Leaves	bloating, indigestion	Infusion	Oral	1 day	0.17	Insomnia
BRL 36	*Moringa ovalifolia Dinter & A. Berger*	Moringaceae	Omoringa	Tree	Leaves, seeds	diarrhea, stomach pain, stomach discomfort	Infusion	Oral	1-2 days	0.17	high blood pressure

BRL 37	*Ziziphus mucronata Wild*	Rhamnaceae	Omusheshete	Tree	Roots, twigs	stomach discomfort, diarrhea	Decoction	Oral	1 -7 days	0.43	nose bleed
BRL 40	*Terminalia sericea Burch ex DC.*	Combretaceae	Omugolo	Shrub	Roots, twigs	diarrhea, stomach pain	Decoction	Oral	7 days - 1 year	0.91	mental illness
BRL 42	*Pollichia camestris Aiton*	Caryophyllaceae	Onona yaasita	Shrub	Leaves	stomach pain, bloating	Macerations	Oral	3 days	0.43	None
BRL 34	*Albizia anthelmintica (A. Rich) Brongn*	Fabaceae	Omupopo	Tree	Bark, roots, flowers, seeds	stomach worms, stomach pain diarrhea	Decoction	Oral	1-7 days	0.91	None
BRL 39	*Colophospermum mopane (J. Kirk ex Benth) J.Kirk ex J, Leonard*	Fabaceae	Omusati	Tree	Twigs, gum, roots, twigs, leaves	stomach pain, diarrhea, stomach worms	Maceration and Chewing	Oral	1 -7 days	0.78	Eye illness, wound healing, gum infection, flu, stop bleeding
AI 02	*Boscia albintrunca*	Capparaceae	Omunkunzi	Tree	Roots, twigs leaves	stomach pain, diarrhea,	Decoction	Oral	7 days	0.35	eye infection
AI 05	*Sesamum capense Burm.f.*	Pedaliaceae	Enanza	Shrub	Leaves	stomach pain, constipation	Maceration	Oral	1hour	0.57	None
BRL 99	*Lantana camara*	Verbenaceae	Iikulya yaanahambo	Shrub	Leaves, seeds, roots, stem	diarrhea, stomach pain	Decoction	Oral	3 days	0.91	Fever

BRL 100	NSI	NSI	Endombo	Shrub	Leaves	diarrhea, stomach pain	Decoction	Oral	1 day	0.13	None
BRL 101	NSI	NSI	Omutuutuu	Tree	Leaves	stomach pain	Chewing	Oral	1-3 days	0.09	None
BRL 102	NSI	NSI	Ekwaava	Tree	Leaves	Diarrhea	Chewing and Decoction	Oral	1-3 days	0.09	Cough
BRL 103	NSI	NSI	Ompilu	Shrub	Roots	stomach discomfort	Decoction	Oral	1-2 weeks	0.04	None
BRL 104	NSI	NSI	Dhingila	Shrub	Roots	stomach discomfort	Decoction	Oral	1-2 weeks	0.09	None
BRL 104	NSI	NSI	Okaneema	Tree	Leaves	Stomach discomfort	Chewing and Decoction	Oral	1-2 weeks	0.09	Cough

Footnotes: BRL: Biomedical Research Lab; AI: Albertina Iikasha, NSI: Not Scientifically Identified

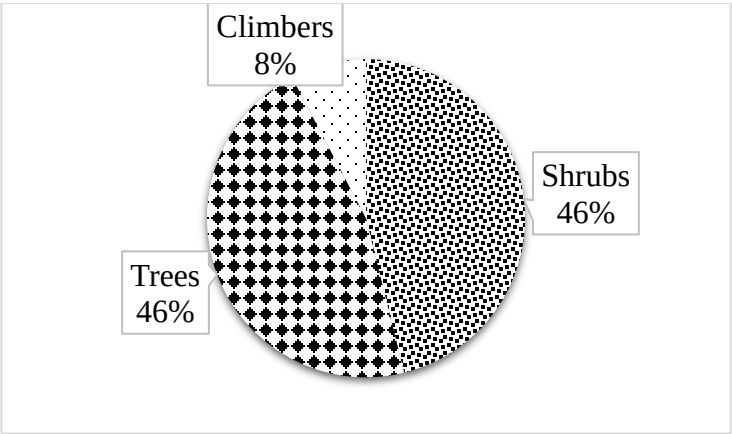

Figure 2: The life form of medicinal plants used to manage gastrointestinal conditions

3.4.3 Methods for preparing gastrointestinal remedies

Medicine for gastrointestinal conditions was prepared in the forms of decoctions, infusions, Chewing, and maceration. Decoction (54%) is the most preferred method for preparing medicine (Figure 3). The maceration technique of herbs involves steeping and fermenting medicinal plants to make medicinal beverages. Oral administration was the only route used in the administration of medicine when treating different gastrointestinal conditions (Table 3). Different plant parts such as twigs, leaves, roots, gum, flowers, and whole plants are used to prepare the medicine. For most plants, all parts (whole plant, 29%) can be used in preparing medicine (Figure 5). Most of the knowledge holders interviewed made use of *G. tenax, T. sericea, A. anthelmintica, L. camara,* and *C. tridens* (Figure 4 a-e) to treat and manage different gastrointestinal conditions with RFC values of 0.71, 0.91, 0.91, 0.91, and 0.71 respectively as depicted in Table 3. Other studies have identified decoction, infusion, and maceration as the most used techniques for preparing medicine in the traditional setting (Abubakar and Haque, 2020). Although the use of these medicinal plants is documented in some parts of Namibia, there are differences in the part

used, preparation methods of medicine, routes of administration, and recovery periods within districts.

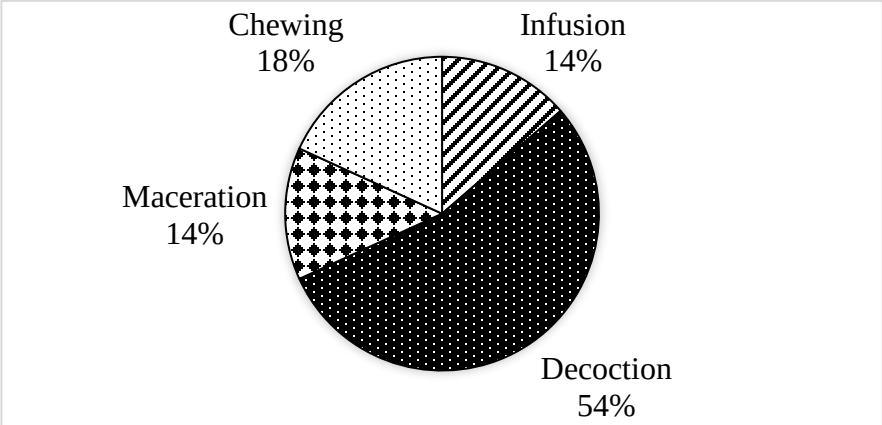

Figure 3: Mode of preparation reported to manage gastrointestinal conditions

Figure 4: Medicinal plants that are frequently used in Iikokola village to treat gastrointestinal conditions in their vegetative stages. (a) *G. tenax* (b) *L. camara* (c) *C. tridens* (d) *T. sericea* (e) *A. anthelmintica.* Photographed in the Omusati region of Namibia in April 2017 (Shatri and Mumbengegwi, 2017).

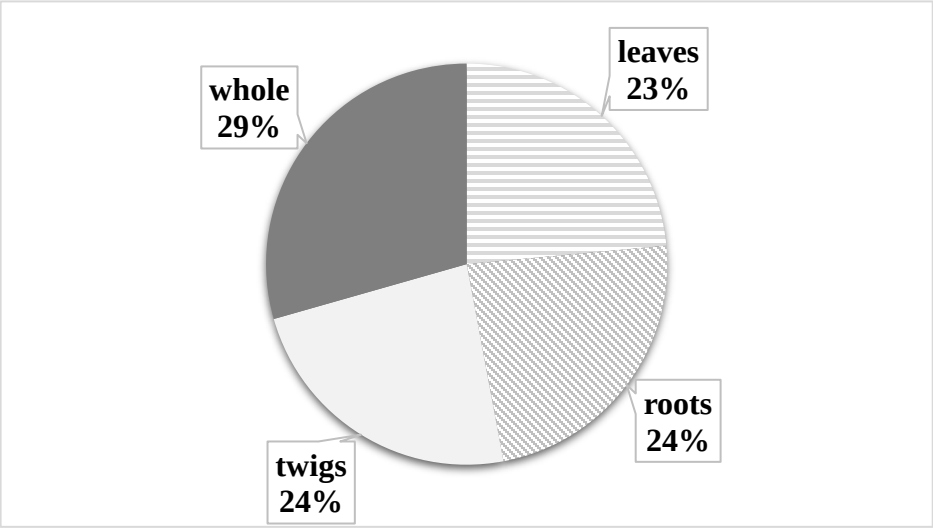

Figure: 5. Plant parts used in medicine preparation for gastrointestinal conditions

3.4.4 Qualitative and quantitative phytochemical analysis

Screening of phytochemical compounds in aqueous and organic extracts of the five plants with higher RFC values reported in this study was determined by the TLC method. The presence of classes of phytochemical compounds was qualitatively described as high, moderate, or low based on the intensity of color on TLC plates. Organic extracts mimicking the maceration and fermentation technique used in the traditional brewing of *G. tenax, T. sericea, C. tridens, A. anthelmintica,* and *L. camara* extracts showed a higher to the moderate presence of compounds such as coumarin, saponin, anthraquinones, flavonoids, and tannins. The presence of alkaloids was low in most organic extracts. Steroids were only observed in the seed extract of *T. sericea* (Tables 4 and 5). Unlike organic extracts, coumarin, saponin, flavonoid, tannins, and anthraquinones were low in all aqueous extracts. Triterpenoids and steroids were absent in all aqueous extracts as

depicted in Table 4. Alkaloids such as sanguinarine, berberine, tomatidine, reserpine, and piperine are linked to the antimicrobial and anti-inflammatory properties of plants (Barbieri *et al.,* 2017). Although the composition of phytochemical compounds in the extract plays a significant role in the effectiveness of the plant extract in curing a disease, the form of medicinal extracts delivery (whole herbs, such as herbal tea or fresh juice) also significantly affects the treatment success (Nagalingam, 2017). Even though the aqueous extracts prepared in this study showed limited phytochemical compounds ethnomedicinally prepared decoctions and infusions of therapeutic effects in Iikokola village are reported to have therapeutic properties within the gastrointestinal tract. Hence further studies should be conducted to determine the biological effects of the prepared extracts.

Table 4: Screening of phytochemical compounds in aqueous extracts using thin layer chromatography method

Plants name	Coumarin	Saponin	Flavonoid	Alkaloid	Steroid	Tannin	Anthraquinone	Triterpenoid
T. sericea **seeds**	+	+	+	++	-	+	+	-
Twigs	+	-	+	-	-	+	+	-
A. anthelmintica	+	-	+	-	-	+	+	-
roots	+	-	+	+	-	+	++	-
twigs								
G. tenax **roots**	+	+	+	-	-	+	+	-
Twigs	+	++	+	-	-	+	+	-
L.camara								
twigs	+	+	+	+	-	+	+	-
seeds	++	++	+	+	-	+	+	-
C. tridens **the whole plant**	+	+	+	+	-	+	-	-

Key: - absent; +: low presence; ++: moderate presence; +++: higher presence

Table 5: Screening of phytochemical compounds in organic extracts using thin layer chromatography method

Plants name	Coumarin	Saponin	Flavonoid	Alkaloid	Steroid	Tannin	Anthraquinone	Triterpenoid
T. sericea **seeds**	+++	++	+++	+	+	+++	+	-
twigs	++	++	++	+	-	+++	-	-
A. anthelmintica								
roots	++	++	+++	+	-	++	+	-
twigs	++	++	++	++	-	++	+	-
G. tenax **seed**	+++	+++	+++	+	-	+++	++	+
twigs	+++	+++	+++	+	-	+++	++	+
L.camara								
twigs	++	++	++	-	-	+++	++	-
seeds	+++	++	+++	+	-	+++	++	-
C. tridens **the whole plant**	++	+	++	+++	-	+	+	++

Key: - absent; +: low presence; ++: moderate presence; +++: higher presence

In this study, the total phenol and flavonoid contents were only determined for aqueous and organic extracts that showed a significant presence of phenolic and flavonoid phytochemical compounds. Among the selected plant extracts, methanol extracts of *T. sericea* twigs and seeds showed a higher phenolic content of 276.5 and 248.5 mg GAE/g, followed by twigs and roots of *G. tenax* with phenolic contents of 169.46 mg GAE/g respectively (Figure 6). Among the selected plant extracts, the highest amount of flavonoid content was found in methanol extracts of *T. sericea* seed and twigs (107.8 and 74.03 mg QE/g respectively). *L. camara* extracts showed higher flavonoid contents in the aqueous extract in comparison to organic extracts, while total phenol content was higher

in organic extracts in comparison to the aqueous extract (Figure 6). The extraction procedures and solvents are responsible for dissolving the endogenous compounds of the plants. Moreover, plant components can be polar or nonpolar. However, phenolic compounds are more soluble in polar organic solvents due to the presence of a hydroxyl group, therefore methanol was selected as the extracting solvent in this study. It is widely known that members of different plant families contain secondary metabolites such as tannins, phenolics, and alkaloids that are responsible for their bioactivity (Truong *et al.,* 2019). Studies conducted worldwide showed that secondary metabolites that are reported in this study are responsible for their efficacies in alleviating and treating communicable and non-communicable diseases (Mongalo *et al.*, 2016, Kopaede, Doijad, Magdam, 2019), with phenolic, and flavonoids phytochemicals being associated with therapeutic benefits. The variations in phenolic and flavonoid content in this study compared to those in the literature may be due influenced by the presence of different amounts of sugars, carotenoids, and ascorbic acid, as well as the duration, geographical variation, and the methods of extraction (Aryal *et al.,* 2019). Hence these extracts should be evaluated for biological effects against gastrointestinal pathogens.

Phenolic and flavonoids utilize mechanisms such as cell lysis and disruption of the cytoplasmic membrane by action upon the membrane permeability of diarrheal-causing pathogens, leading to leakage of cellular components and eventually cell death (Tagousop *et al.,* 2018). Studies conducted worldwide showed that secondary metabolites that are reported in this study are responsible for efficacies in alleviating and treating communicable and non-communicable diseases (Mongalo *et al.,* 2016; Aryal *et al.,* 2019). Other studies have also linked compounds such as tannins, saponins, flavonoids, and

terpenoids, to antidiarrheal activity *in vivo* models (Teferi, Abddulwuhab, and Yesuf, 2019). The presence of different phytochemical compounds such as alkaloids, tannins, flavonoids, saponins, anthraquinones, coumarins, and triterpenoids in the extracts of the studied plants and their phytochemicals properties may add to the effectiveness of the phytomedicines. This can enhance positive perceptions of the use of the plants as ethnomedicines for treating diarrhea in the Omusati region, contributing to a higher FIV. The presence of different phytochemical compounds in extracts prepared from *G. tenax, T. sericea, C. tridens, A. anthelmintica*, and *L. camara* add value to their popular ethnomedicinal uses as alternative and complementary medicines in treating diarrhea in Omusati region.

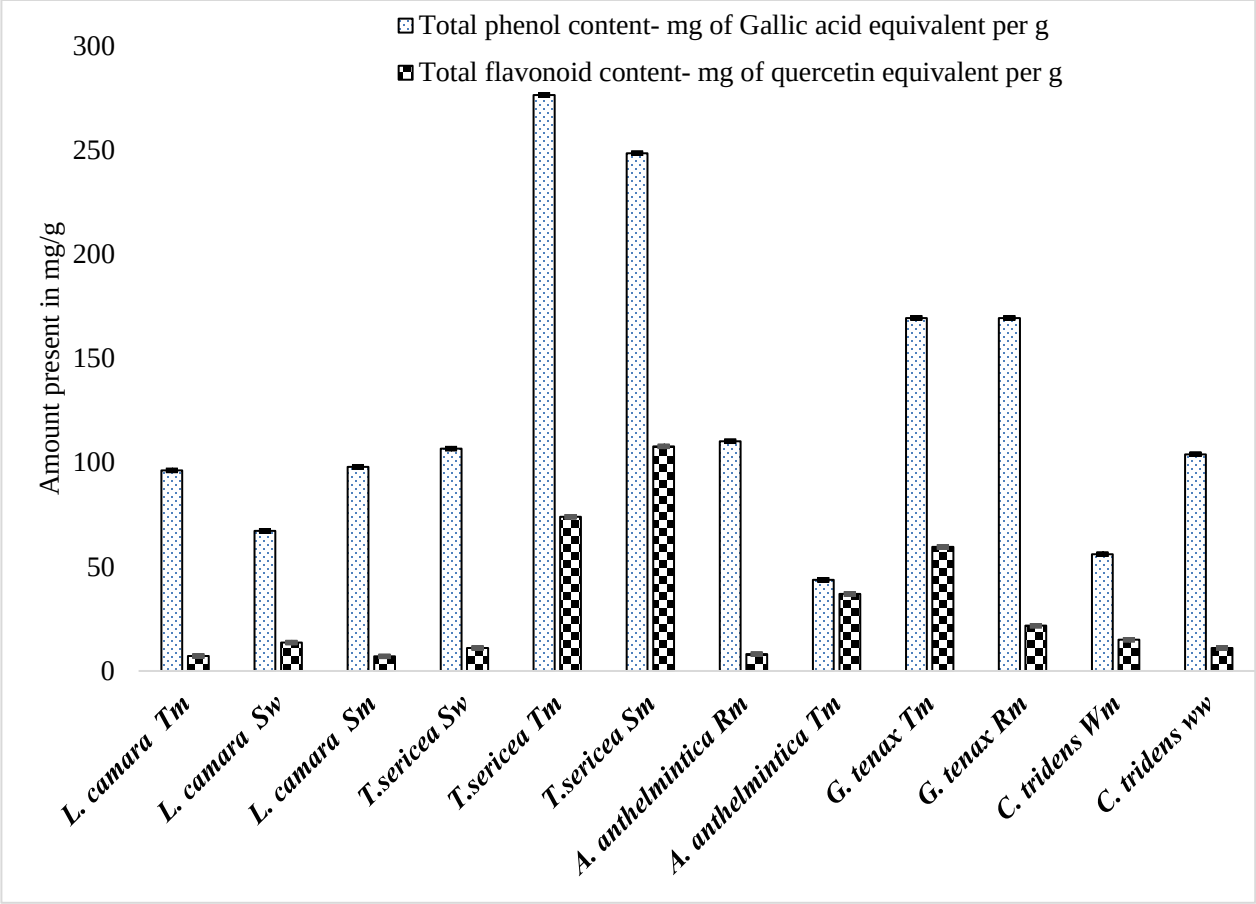

Figure 6: A comparison of total phenol (mg GE/g) and flavonoid content (mg QE/g) in different plant extracts. Data represented as mean ± standard deviation, Mean± SD, n= 3. Tm: Twig methanol extract, SW: Seed Water extract, Sm: Seed methanol extract, Tm: Twig methanol extract, Rm: Root methanol extract, Wm: Whole plant methanol extract, Ww: Whole plant water extract**.**

3.5 Conclusion

This study is the first report on the use of medicinal plants used to treat gastrointestinal conditions in northern Namibia, it revealed that the area has medicinal plants which are still commonly used for medicinal purposes by the local people. Thus, it is important to document and reconstitute the remaining indigenous medical practices which exist in this area and other parts of Namibia with reduced access to health facilities. These communities are more likely to rely on ethnomedicine for primary healthcare and hence

their knowledge can be documented and preserved for future generations. The results indicate that traditional healers used 19 species of medicinal plants to cure gastrointestinal disorders and other ailments of which the gastrointestinal uses of 6 species are novel to this study and have not been reported in any other regions in Namibia. Most of the cited plants require a more in-depth evaluation of their pharmacological activities to fully understand how they can be used to treat gastrointestinal diseases. Data on efficacy and safety will also contribute to the promotion of their mainstream use as complementary and alternative medicine. This study also provided the basis for the safeguarding of the knowledge on the uses of medicinal plants for future uses toward plant-based next-generation drug development. Qualitative and quantitative phytochemical analysis revealed the presence of phytochemical compounds with antidiarrheal properties. Hence these plants should be screened for their efficacies in inhibiting different diarrheal pathogens such as *Salmonella, E. coli, Shigella*, *Clostridium* species, and viruses such as the *Rotavirus* and *Adenovirus* that are known to be responsible for most reported diarrhea cases globally. This study contributed to the documentation and validation of the phytochemicals found in *G. tenax, T. sericea, C. tridens, A. anthelmintica,* and *L. camara* from Iikokola.

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CHAPTER 4: EVALUATION OF ANTIBACTERIAL ACTIVITIES AND CYTOTOXICITY OF SELECTED MEDICINAL PLANTS USED IN THE OMUSATI REGION OF NAMIBIA, FOR MANAGEMENT OF GASTROENTERITIS, AGAINST A PANEL OF MULTIDRUG-RESISTANT CLINICAL AND REFERENCE STRAINS OF *SALMONELLA* AND *ESCHERICHIA COLI*

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4.1 Abstract

Acute gastroenteritis and antibiotic resistance are major global health concerns accounting for over 1.3 million mortality cases in pediatrics globally. While phytomedicines could be the source of next-generation complementary and alternative medicines for gastroenteritis, there is a gap in knowledge regarding the efficacy and safety of most medicinal plants. The study aimed to evaluate the antibacterial and cytotoxicity of selected medicinal plants used in Namibia to manage gastroenteritis. Aqueous and methanol extracts were prepared from *Lantana camara, Grewia tenax, Corchorus tridens*, *Albizia anthelmintic*, and *Terminalia sericea*. Agar disc diffusion and resazurin-based microtiter

dilution assay were used to determine the antibacterial activity. Antigram assay was used to determine the susceptibility of clinical and reference gastrointestinal pathogens to 11 clinically used antibiotics. NiH/3T3 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay were used for the cytotoxicity test. A 70% multidrug antibiotic resistance was observed among clinical isolates of *Salmonella* and *Escherichia coli*. Meanwhile, *L. camara* methanol seed extracts showed higher activity against clinical strains of *E. coli* and *Salmonella* (20.67± 1.53 and 21.67± 0.58 mm respectively), with MICs between 31.25 and 62.5 µg/ml. The IC_{50} of all extracts was ≥65.85.00± 0.58 µg/mL, which is > 20 µg/mL set as the indicator for cytotoxicity in plant extracts. The study showed that methanol extracts of *L. camara, C. tridens*, and *T. sericea* were effective in eliminating multidrug-resistant clinical *Salmonella* and *E. coli*. Methanol extracts did not show significant toxicity to 3T3 cells.

Keywords: Multidrug-resistance; Antibacterial; Cytotoxicity; Gastroenteritis

4.2 Background

Diarrheal diseases are a major global health concern in pediatrics accounting for over 1.3 million mortality cases in children under the age of 5 annually (Giannattasio, Guarino, and Lo Vecchio, 2016; Ochoa, 2011). Rotavirus is the most common cause of acute diarrhea among children, however responsible for 25 million outpatient visits and more than 2 million inpatients every year. Important bacteria such as *Salmonella, Shigella, Yersinia, Campylobacter,* and *Escherichia coli* are responsible for a significant number of deaths among children; especially in immunocompromised pediatrics especially in Sub- Saharan Africa (Mokomane *et al.*, 2018; Fletcher, Stark, and Ellis, 2011; Iikasha,

Quaye, and Mumbengegwi, 2020). In a study consisting of 412 patients with gastroenteritis, 32.3% of the cases were due to bacterial gastroenteritis (Sgen *et al.,* 2016).

Bacterial diarrhea occurs mainly via the ingestion of contaminated food or water. *Salmonella* invades the mucosal membrane of the distal ileum and proximal colon. *Salmonella* can survive within a hostile gastric environment with low pH and avoid lysis by bile salts in the upper small intestine (Crum-Cianflone, 2018). It specifically colonizes the M-cells that are located at the top of the lymphoid follicles of Peyer's patches (Mabbott, 2013). If not eliminated, the microbes then penetrate the sub-mucosa and enter the bloodstream via the lymphatics. Transient bacteremia follows and the *Salmonella* spread to the endothelium of the liver, spleen, bone marrow, gall bladder, and kidneys. The organisms will then re-enter the intestine from the gallbladder resulting in the inflammation of the Peyers' patches and ulceration (Ugboko and De, 2014). *E. coli* invade the mucosal membrane of the small intestine where it binds to the host cell membranes. A particular strain of *E. coli* known as *E. coli* O157:H7 is reported to cause severe intestinal infection in humans. *E. coli* O157:H7 can be differentiated from other *E. coli* by the production of a potent toxin that damages the lining of the intestinal wall causing bloody diarrhea (Gambushe, Zishiri, Zowalaty, 2022). It is also known as an enterohemorrhagic *E. coli* infection which is a Shiga toxin-producing *Escherichia coli* strain (Kaper, Nataro, Mobley, 2004). Shiga toxin produced by *E. coli* O157:H7 gets endocytosed and translocated through the cell in a process involving trans-Golgi vesicular transport leading to systemic complications such as hemolytic uremic syndrome, which can lead to kidney failure and death (Nataro and Kaper, 1998; Ameer, Wasey, and Salen, 2021).

In many cases, oral rehydration is recommended to manage acute diarrhea in children instead of antibiotic therapy (Bruzzese, Giannattasio, Guarino, 2018). However, in immune-compromised children with persistent diarrhea treatment using probiotics and antibacterial agents is highly recommended (Pavlinac, Tickell, Walson, 2015; Centers for Disease Control and Prevention, 2022). The use of antibiotics and antidiarrheal agents is fraught with complications due to undesired side effects of the treatments that include vomiting, nausea, severe diarrhea, and loss of appetite largely due to large dosages administered. All those challenges limit the effectiveness of existing antibiotics and antibacterial agents and have resulted in increased mortality and morbidity cases due to acute diarrhea, especially in children and immunocompromised patients (Prabhuswamy, Kuruni, and Mallikarjun, 2016). Moreover, antibiotic resistance has become a global health crisis estimated to be responsible for 700,000 deaths annually worldwide (World Health Organisation, 2019). Higher rates of antibiotic resistance to first-line antibiotics such as amoxicillin, erythromycin, gentamycin, and ampicillin in gastrointestinal pathogens are reported daily (Fair and Tor, 2014). Hence, the World Health Assembly adopted a Global Action Plan on antimicrobial resistance in 2015, with national governments in more than 120 countries acting on the challenges presented by antimicrobial resistance (Baekkeskov *et al.,* 2020; Centers for Disease Control and Prevention (CDC), 2016). Limitations presented by existing antibiotics and antibacterial agents call for alternative medicine with fewer complications and significant therapeutic effects.

In recent years, there have been an increasing number of studies to discover new bioactive compounds of plant origin with the hope to control antibiotic-resistant bacteria

(Subramani, Narayanasamy, and Feussner, 2017). This is because medicinal plants have been used in all age groups for a long period, with 80% of populations in developing countries being reported to rely on medicinal plants as an alternative treatment option for diarrhea and other ailments (Njume and Goduka, 2012; Ugboko *et al.,* 2020). However, despite the long history of use, there is limited scientific data to validate the efficacy and safety of medicinal plants especially those used of plants to manage diarrhea. The escalating global cases of antibiotic resistance especially among gastrointestinal bacteria require alternative treatment options for diarrhea from bacteria such as *Salmonella, Shigella, Campylobacter,* and Verotoxigenic *Escherichia coli* species (Global Salm-Surv; 2003), especially in African countries such as Nigeria, Namibia, Ethiopia, Chad, Mali and the Democratic Republic of Congo, (Iikasha *et al.,* 2020; Global Salm-Surv; 2003).

Cytotoxicity evaluation of medicine is important as it provides information on the cell growth, viability, morphology, effect of the pharmaceutical product, the safe dose, and potential toxicity profile. Currently, in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, the neutral red uptake (NRU), resazurin reduction (RES) and sulforhodamine B (SRB) assays are commonly used methods (van Tonder, Joubert, and Cromarty, 2015). MTT is mostly used because of its ease and rapidity of performance, reproducibility of the results, and observed clinical correlation between in vitro and in vivo testing (Spino, 1995).

Hence, the objective of this study was to determine the antibacterial activity of *T. sericea* (BRL 40), *L. camara* (BRL 99), *G. tenax* (BRL 33), *C. tridens* (BRL 30), and *A. anthelmintica* (BRL 34) (that where identified in chapter 3) and the antigram activity of selected antibiotics against clinical isolates of *Salmonella spp* and Verotoxigenic

Escherichia coli 157:H7 and reference strains of *Salmonella Typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922 as well as to determine the cytotoxicity of the selected extracts to the of NiH/3T3 normal mouse embryo fibroblast cells.

4.3 Materials and methods

4.3.1 Plant material

T. sericea (twigs and seeds), *C. tridens* (whole), *L. camara,* (twigs and seeds), *G. tenax* (roots and twigs*)*, and *A. anthelmintica* (twigs and roots*)* plant materials were selected for this study based on their frequency of ethnomedicinal use by people in Iikokola village, Omusati region in Namibia to manage different gastrointestinal conditions including gastroenteritis. The identification of the plants was conducted by the National herbarium of Namibia (permit number: 2221/2017) in Appendix B, F and G. Ethical clearance (Appendix A) to conduct this study was obtained from the University of Namibia's ethical committee (SOS-0039).

4.3.2 Preparation of crude extract

T. sericea (twigs and seeds), *C. tridens* (whole), *L. camara* (twigs and seeds), *G. tenax* (roots and twigs*)*, and *A. anthelmintica* (twigs) plant materials were collected, washed with distilled water, and air-dried in the shade for 4 weeks. The dried plant materials were ground to a fine powder using an industrial grinder. For the preparation of organic extracts, 20 000 µg of the plant materials were added to 200 ml of methanol and mixed on a shaker for 48 hours at room temperature for maceration. Aqueous extracts were prepared by adding 20 000 ml of the plant materials to 200 ml of distilled water and keeping the mixture in a water bath set at 60 ºC water for 4 hours. All extracts were filtered

using Whatman no 1 filter papers and the filtrates were concentrated by rotary evaporation at 40 °C and then freeze-dried to form a powder. The powdered extracts were stored at -20ºC. The percentage yield was calculated using the formula:

$$\% \text{ Yield} = \left(\text{Mass of plant}\frac{\text{extract}}{\text{Mass}}\text{of plant material}\right) \text{x } 100$$

4.3.3 Clinical isolates and reference strain

Four bacterial isolates were used in this study. Clinical *Salmonella* spp and *E. coli* 157:H7 VTEC were isolated from stool samples as described by Iikasha *et al* (2020); while the reference strains of *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922 were used. The bacterial isolates were used for the comparative evaluation of the antibacterial activity of organic and aqueous crude plant extract as well as for the antibiogram assay.

4.3.4 Preparation of bacterial culture

Using aseptic techniques, test tubes of Muller Hinton broth were inoculated with the reference and clinical bacterial isolates respectively. The inoculated broths were incubated for 18 h at 37° C. After incubation, the turbidity of the bacteria cultures was adjusted to match the 0.5 McFarland standard by serially diluting the cultures with Muller Hinton broth. The viability graph was used to calculate the actual number of colony-forming units. The dilution factor was calculated, and the dilution was performed to obtain a final concentration of 5 x 10^6 CFU/ml.

4.3.5 Antibiotic susceptibility testing of clinical and reference strains of *Salmonella and E. coli*

Antibiotic susceptibility tests of the clinical isolates and reference strains; and the antibacterial activity of each plant extract was determined using the modified Kirby-Bauer disc diffusion method in agreement with the guidelines of the National Committee for Clinical Laboratory Standards (2003). The antibiotics used for the antibiogram assay included: ciprofloxacin (5 µg/ml), erythromycin (15 µg/ml), tetracycline (30 µg/ml), vancomycin (30 µg/ml), cephalothin (30 µg/ml), ampicillin (10 µg/ml), sulphamethoxazole (25 µg/ml), gentamicin (10 µg/ml), penicillin G (10 µg/ml), and amoxicillin (10 µg/ml). The plant extract (1000 µg) was re-dissolved in 2 ml of methanol and vortexed. Sterile Whatman filter paper discs were placed in falcon tubes containing the plant extracts of the concentration of 500 µg/ml and macerated for 24 hours at 4°C. Petri dishes containing 8 ml of solidified Mueller-Hilton agar were inoculated with 10 µl of the inoculums containing clinical isolates and reference strains of *Salmonella* and *E. coli* respectively. The discs were allowed to warm to ambient temperature before application to prevent condensation. The impregnated discs and antibiotic discs were placed on Mueller-Hilton agar plates respectively. The plates were allowed to stand for 30 minutes at 4°C to allow plant extracts and antibiotics to diffuse into the media and then incubated at 37 °C for 24 hours. Distilled water and 99% methanol were used as negative controls.

4.3.6 Validation of the resazurin-based Microtiter Dilution Assay

To establish the accuracy of this method, the performance of 3 standard antibiotics namely tetracycline which is bacteriostatic as well as gentamycin, and ciprofloxacin which are bactericidal against reference strains of *Salmonella Typhi* ATCC 13311 and *Escherichia coli* ATCC 25922 was determined. The MIC values obtained were compared with those

published by the Global Salm-Surv, 2013 and the National Committee for Clinical Laboratory Standards, 2003). The MIC was determined based on the color change observed. With resazurin-based MIC assay, active living cells cause a reduction of resazurin (purple-blue) to resorufin (pink-colorless).

4.3.7 Resazurin-based Microtiter Dilution Assay to determine the minimum inhibitory concentrations of the plant extracts

Resazurin dye (600 mg) was dissolved in 80 mL of sterile distilled water. A vortex mixer was used to homogenize from the Resazurin dye solution. Under aseptic conditions, 96 well microtiter plates were used for the Resazurin-based Microtiter Dilution Assay. For each plate, the first row of the microtiter plate was filled with 100 µl of organic or aqueous plant extract stock solution of 500 µg/ml. All the wells of microtiter plates were filled with 100 µl of nutrient broth. Two-fold serial dilution (throughout the column) was achieved by starting to transfer 100 µl from the first row to the subsequent wells in the next row of the same column so that each well has 100 µl of plant extracts in serially descending concentrations. Exactly, 10 µl of resazurin solution as an indicator was added to each well. Finally, a volume of 10 µl was taken from bacterial suspensions of clinical *Salmonella* spp and *E. coli* 157:H7 VTEC; and laboratory isolates of *Salmonella Typhi* ATCC 13311 and *Escherichia coli* ATCC 25922 (5×10^6 CFU/ml) was added to each well respectively. To avoid the dehydration of bacterial culture, each plate was covered loosely with the plate lid. Each microtiter plate had a set of 3 controls: (a) a column with tetracycline (30 µg) as a positive control, (b) a column with all solutions except for the test extract, and (c) a column with all solutions except bacterial solution replaced by 10 µl of nutrient broth. The plates were incubated at 37° C for 24 hours. The color change in

the well was then observed visually. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. The dye remains purple in the absence of bacteria growth (non-fluorescent) however when there is bacteria growth the dye becomes pink and fluorescent as it is reduced to resorufin by oxidation-reduction within viable cells. Resorufin is further reduced to hydro-resorufin (uncolored). Any color change observed from purple to pink or colorless was taken as positive. The lowest concentration of plant extract at which no color change was recorded meaning at which there was no bacteria growth observed was the MIC value. All the experiments were performed in 3 experimental repeats. The average values were calculated for the MIC of the test material (Gahlaut and Chhillar, 2013; Elshikh *et al.*, 2016).

4.3.8 Cytotoxicity assay

4.3.8.1 Culturing of normal mouse embryo fibroblast cells (NiH/3T3) cells

The 3T3 cells used in this study were initially cultured and maintained as described by (Pour, Latha, and Sasidharan, 2011), with minor modifications. Frozen cells were removed from −-80 °C, thawed at 37 °C, and grown for 1 week at 90% Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal calf serum FSC and 1% streptomycin at 37 °C in 95% humidity with 5% CO2. The cultured cells were viewed under the inverted microscope and the confluence of the cell monolayer was monitored until it was about 80-90%. Under sterile conditions in the biosafety cabinet, the flasks with over 90% confluence were selected for further analysis. The culture media was aspirated from the cells and washed with warm sterile phosphate buffer saline (PBS). After aspirating out PBS, 10 % of the enzymatic digestion trypsin in PBS was added to

each flask and the flasks were incubated in the CO_2 incubator at 37 °C for 3 minutes to allow the cells to detach from the flask surface. Scrapers were used to gently stripe off the cells from the flask surface. Briefly, fresh syringe-filtered media was added to each flask with cells and swirled gently. The cell suspensions were transferred to a falcon tube and centrifuged for 7 minutes using a Mikro 200 & 200R centrifuge at 1425 xg relative centrifugal field (RCF). After discarding the media, the pellet was resuspended in fresh media and mixed gently. Cells were sub-cultured once a week and used before the 10^{th} passage.

4.3.8.2 Cell count and seeding density

Briefly, 200 ul of trypan blue stock solution was transferred to a 15 ml falcon tube and 9.8 ml of 1% acetic acid (working solution). Exactly 100 ul of the cell suspension was transferred into a 1.5 ml falcon tube and add 100 ul of trypan blue (Zhang, Lin, and Ye, 2018). Briefly, 10 ul of the mixture was loaded onto the haemocytometers and viewed at the lower magnification and the cells were counted inside each of the 4 large corner squares (the total number of cells counted should be ≥50). The cell density was calculated using the equation below:

$$\text{Total}\,\frac{\text{cells}}{\text{ml}} = \text{Total cell count x}\left(\text{Dilution}\,\frac{\text{factor}}{\text{Number}}\,\text{of squares}\right)\text{x } 10000$$

Using the obtained total cell count, and the desired volume the seeding density was calculated. The cells were seeded at $2.85x10^5$ per well. Briefly, 100 ul of the $2.85x10^5$ per ml cell suspension was seeded onto all the 96-well microtiter tissue culture plates except for the wells used for blank. The 96 well plates were incubated at 37 °C in the CO_2

incubator for 48 hours to allow 80-90% confluence in the 96-well microtiter tissue culture plates.

4.3.8.3 Extracts preparation

For each extract, a stock solution (SS) of 10 mg/ml concentration was prepared in 1:9 of dimethylsulphoxide (DMSO) and media (10 ml) respectively, and vortexed for 5 minutes at room temperature. Exactly l mg/ml concentration was prepared by adding 100 µl of the SS to 900 µl of the fresh media. A desired initial working concentration of 100 µg/ml was prepared by adding 20 µl of the 1mg/ml concentration to 980 µl of the fresh media. The solution was then syringe filtered. Serial ten-fold dilutions were made from SS to give working concentrations of 100- 3.125 µg/ml. Confluent monolayers of the cells were grown in 96 well-microtiter plates for 48 hours. Cells were incubated with various concentrations of the test extracts in triplicate at 37 °C in a CO_2 incubator for 48 hours. The negative control was performed using a growth medium alone instead of plant extract, while Triton-x was used as the positive control.

4.3.8.4 Determination of cell viability by MTT assay

Cell viability was examined by the ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (Sigma, Chem, St. Louis, MO), by the mitochondrial enzyme succinate dehydrogenase following the procedure as described (Chen, Yu, and Luo, 2016) with minor modifications. After 48 hours, supernatants were removed from the wells and 30 µl of 5 mg/mL MTT solution was immediately put into each well, followed by an incubation period of 2 hours at room temperature in the dark. The plates were incubated for 2 hours at room temperature.

Afterward, the media in the plates was carefully discarded. For dissolving formazan blue crystals, 100 µl of dimethyl sulfoxide (DMSO) was added to each well and incubated at room temperature for 15 minutes in the dark. The plates were placed in the spectrophotometer and shaken for 5 minutes. The optical density was determined at 570 nm using a Uv -vis multi-well spectrophotometer JASCO. The percentage of cell growth inhibition was calculated based on a comparison with the untreated cells.

The percentage of cell viability was calculated as per the following formula:

$$\text{Cell viability (\%)} = ((\text{Absorbance of treated cells} - \text{Absorbance of blank})/ (\text{Absorbance of untreated cells} - \text{Absorbance of Blank})) \times 100$$

The 50% Inhibitory concentration (IC_{50}) was defined as the extract concentration required for the reduction of cell viability by half. The IC_{50} value and the standard error mean (SEM) were calculated using a non-linear regression curve contained in excel. (Concentrations 100- 3.125 µg/ml).

4.4 Statistical analysis

All experiments were done in experimental triplicates and statistical analysis was performed employing Graph Pad Prisms software version 7.0. Comparison between groups was done using Two-way ANOVA, followed by the Bonferroni post-tests test in which the $P < 0.05$ were considered significant.

4.5 Results and Discussion

4.5.1 Effect of extracting solvent on the percentage yields of crude extracts

Organic and aqueous plant extracts were prepared from selected four plants using methanol and distilled water respectively. The percent extract yield of organic and

aqueous plant extracts varies between 1.0 to 13.5% (Table 1). In most cases, the amount of residue extracted with methanol is higher than that of distilled water. However, the percentage yields for *L. camara* organic and aqueous seeds and twigs extracts and organic and aqueous twigs extracts are equal respectively. Seed extracts yielded higher than twigs and root extracts (Table 1). Studies have linked the percentage yield of the crude extract to its efficacy and bioprospecting properties (Isaiah, Arun, and Senthamizh, 2016).

Table 1: Percent (%) yield of various organic and aqueous plant extracts

Sample number	**Plant name**	**Part used**	**The percent yield of plant extract**	
			Aqueous extracts	Organic extracts
BRL 33	*G. tenax*	Roots	1.0	6.5
		Twigs	3.0	3.0
BRL 34	*A. anthelmintica*	Seeds	1.5	8.0
		Twigs	9.5	1.5
BRL 40	*T. sericea*	Seeds	10.0	12.0
		Twigs	4.0	8.5
BRL 99	*L. camara*	Seeds	13.5	13.5
		Twigs	12.5	12.5
BRL56	*C. tridens*	Whole	1.0	8.0

4.5.2 Antibacterial activity of aqueous and organic extracts

The antibacterial activity of organic and aqueous extracts of *G. tenax, A. anthelmintica, T. sericea, L. camara,* and *C. tridens* was assessed against clinical and reference strains of *E. coli* and *Salmonella*. Organic extracts showed higher and broad-spectrum antibacterial activity in comparison to aqueous extracts (Table 2 and Table 3). Higher antibacterial activity was observed in organic seed extracts of *L. camara* against both clinical and reference strains of *E. coli* (20.67± 1.53 mm and 22.00± 1.00 mm) and *Salmonella* species (21.67± 0.58 mm and 20.33± 0.58 mm) respectively (Table 3). The *L. camara* (seeds) and *C. tridens* were the only aqueous extracts that showed antibacterial activity against clinical isolates of *Salmonella spp* (11.33± 0.58 mm) and *E. coli* 157:H7 VTEC (11.67±0.58 mm) respectively (Table 2). *T. sericea* twigs and seeds, *L. camara* seeds, and *C. tridens* also showed antibacterial activity against reference strains of *E. coli,* with higher activity depicted by *L. camara* seeds (12.33± 0.58 mm) and *C. tridens* whole plant (12.00± 1.00 mm). Among other, aqueous extracts, *T. sericea* twigs, and *L. camara* seeds, were the only aqueous extracts that showed antibacterial activity against the reference strain of *S. Typhurium* (Table 2).

The results of this study help to validate the efficacy of 18 extracts prepared from medicinal plants used as alternatives to treat gastrointestinal conditions in Northern Namibian. In this study, *L. camara* organic extracts displayed higher antibacterial activity against *Salmonella* and *E. coli* strains. These findings are consistent with that of (Sharma *et al.,* 2016), who reported significantly higher activity in extracts of *L. camara* and *C. tridens* respectively. Moreover, other studies have been conducted globally on the efficacy of *T. sericea* and *G. tenax* against diarrheal pathogens, but none of these studies tried to

compare the efficacy in clinical and reference strains. This is significant because, as observed in this study, there is a significant difference in the efficacy of extracts against clinical and reference strains, hence, the use of laboratory isolates alone will not fully reflect the efficacy of the extract in clinical isolates. Although reference strains are useful in susceptibility tests, their uses should not replace clinical isolates, and this is supported by the findings observed in this study. This is important, especially when analyzing the antibacterial effects of extracts that are being considered in clinical applications.

Isaiah *et al*., 2016 reported that *C. tridens* had antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Klebsiella aerogenes*, and *Escherichia coli*, the findings of this study also demonstrated broad-spectrum antibacterial activity against clinical and reference strains of *Salmonella* and *E. coli* was observed in its aqueous and organic extracts. Based on statistical analysis, there was a significant difference between the antibacterial activity of *G. tenax* roots, *A. anthelmintica* twigs, and *T. sericea* seeds ($P<0.001$) to clinical and reference strains of *Salmonella*. Meanwhile, *G. tenax* roots, *A. anthelmintic* twigs, *A. anthelmintic* roots, *T. sericea* seeds, and *T. sericea* twigs showed a significant difference in their antibacterial activity against reference and clinical isolates of *E. coli*.

Table 2: Antibacterial activity of aqueous extracts against clinical isolate and reference strains of *Salmonella spp* and *E. coli*, Data presented as mean ± SD, Number of experimental repeats (n) = 3

Plant extracts	**Average inhibition of Clinical *E. coli* (mm)**	**Average inhibition of Reference strain of *E. coli* (mm)**	**Average inhibition of Clinical *Salmonella* isolates (mm)**	**Average inhibition of reference strain of *Salmonella* isolates (mm)**
***G. tenax* twigs**	0±0.0	0±0.0	0±0.0	0±0.0
***G. tenax* roots**	0±0.0	0±0.0	0±0.0	0±0.0
***A. anthelmintica* twigs**	0±0.0	0±0.0	0±0.0	0±0.0
***A. anthelmintica* roots**	0±0.0	0±0.0	0±0.0	0±0.0
***T. sericea* seeds**	0±0.0	8.3±0.58	0±0.0	8±1.0
***T. sericea* twigs**	0±0.0	8±0.0	0±0.0	7.3±0.58
***L. camara* seeds**	9±0.0	12.3±0.58	11±0.58	8.7±0.58
***L. camara* twigs**	0±0.0	10.7±1.2	0±0.0	0±0.0
***C. trides* whole**	11.7±0.58	12.0±1.0	0±0.0	0±0.0
Methanol	0±0.0	0±0.0	0±0.0	0±0.0
Distilled water	0±0.0	0±0.0	0±0.0	0±0.0

Table 3: Antibacterial activity of organic extracts against clinical isolate and reference strains of *Salmonella spp* and *E. coli*, Data presented as mean ± SD, *p<0.05 verse tetracycline, n=3

Plant extracts	**Average inhibition of Clinical *E. coli* (mm)**	**Average inhibition of Reference strain of *E. coli* (mm)**	**Average inhibition of Clinical *Salmonella* isolates (mm)**	**Average inhibition of reference strain of *Salmonella* isolates (mm)**
***G. tenax* twigs**	11.7±1.58*	10.7±0.6*	9.3±0.58*	7±0.00*
***G. tenax* roots**	0±0.00	0±0.00	8.7±0.58*	0±0.00
***A. anthelmintica* twigs**	8.3±0.58*	0±0.00	0±0.00	7.7±0.58*
***A. anthelmintica* roots**	0±0.00	8.7±1.10	0±0.00	0±0.00
***T. sericea* seeds**	0±0.00	14.7±1.58*	17.3±1.53*	15±1.00*
***T. sericea* twigs**	0±0.00	13.3±1.20*	0±0.00	0±0.00

***L. camara* seeds**	20.7±1.58	22±1.00	21.7±0.58	20.3±0.58
***L. camara* twigs**	13.7±1.58*	12±1.00*	13.7±0.58*	14.7±1.15*
***C. tridens* whole**	12.3±1.58	12.3±1.58	13±1.00	12.7±1.15
Methanol	0±0.00	0±0.00	0±0.00	0±0.00
Distilled water	0±0.00	0±0.00	0±0.00	0±0.00
Tetracycline	20±0.60	20.7±0.6	18±0.00	25.7±0.58

4.5.3 The minimum inhibitory concentration of different extracts with antibacterial activity

Results obtained from the validation and quality control assay for MIC by resazurin were within the range of recommended MIC values of the Clinical and Laboratory Standards Institute (2012) as shown in Table 4. Clinical diarrheal pathogens showed higher sensitivity to organic extracts in comparison to reference strains. Among the aqueous extracts, *L. camara* showed the lowest MIC value of 125 µg/ml against the clinical isolate of *Salmonella* (Table 5). Moreover, *L. camara* organic seed extract showed the lowest MIC and MBC of 31.25 µg/ml against clinical *E. coli* 157:H7 VTEC and reference strain of *E. coli* (Table 5). *A. anthelmintica* organic and aqueous extracts showed less sensitivity to clinical and reference strains in this study. Distilled water and 99% methanol that were used as negative controls did not show any inhibitory properties against the test organisms.

While aqueous extracts are normally used in the ethnomedicinal setting to prepare decoctions for treating gastrointestinal, the findings of this study showed minimal efficacy in aqueous extracts in comparison to organic extracts. This could be because bioactive compounds extracted using water require further metabolism to perform bacterial inhibitory properties (Chen *et al.,* 2016). Moreover, while roots are reported to have potent antibacterial activity in other studies, in this study, seeds of the studied plants have shown

potent antibacterial activity with significantly lower MIC values. This is important especially since there is according to the global conservative estimate, the current loss of plant species is between 100 and 1000 times higher than the expected natural extinction rate, and the Earth is losing at least one potential major drug every 2 years (Chen *et al.*, 2016), hence using plant parts that prevent uprooting and killing plants while still offering maximum therapeutic benefits is very important.

Sharma *et al.*, 2016 reported the MIC of *G. tenax* root extracts to be 62.5 and 125µg/ml for aqueous and organic extracts respectively. However, in this study, only the organic twig extract of *G. tenax* showed antibacterial activity with the lowest MIC of 62.5 against clinical *Salmonella* isolate (Table 5). Moreover, other studies have reported MIC values of *L. camara* than the MIC of 31.5 µg/ml that is reported in this study. Opportunistic infections such as gastroenteritis are quite inevitable among children under the age of 5 and immunocompromised patients hence finding alternative medicine to eliminate them is crucial and one way to mitigate the challenge of microbial resistance (Voravuthikunchai, Phongpaichit, and Subhadhirasakul, 2005; Elshikh, Ahmed, Funston, 2016). The findings of this study are the first to use a resazurin assay to evaluate MIC values of methanol and aqueous plant extract. These findings have shown the easy reproducibility and accuracy of MIC evaluation by resazurin and agree with the findings of Elshikh *et al.* (2016). These findings also show the potential of the resazurin–based 96-well plate microdilution method as an interpretational tool for testing the MIC of natural products. It is however observed that tetracycline which was used as a positive control has shown lower MIC compared to all the extracts.

Table 4: Determination of the MIC for ATCC gram-negative strains for quality control purposes

Bacteria	**Antibiotic**	**MIC from this study**	**MIC recommended by CLSI (µg/ml)**
***Salmonella Typhi* ATCC 13311**	Gentamycin	1.0	2-32
	Ciprofloxacin	2	0.03-4
	Tetracycline	3.0	2-32
***Escherichia coli* ATCC 25922**	Gentamycin	1.0	0.25-1
	Ciprofloxacin	0.005	0.004-0.015
	Tetracycline	2.0	0.5-2

Table 5: MIC of organic and aqueous plant extracts against clinical and laboratory isolates of *Salmonella* and *E. coli* by Resazurin microtiter dilution assay.

Plant name	**Part used**	**MIC of plant extract**							
		Aqueous extracts				Organic extracts			
		C.E	R. E	C.S	R. S	C.E	R. E	C.S	R. S
G. tenax	Seeds	-	-	-	-	250±0.0		62.5±0.0	125±0.0
	Roots	-	-	-	-	-	-	-	-
A. anthelmintica	Twigs	-	-	-	-	500±0.0	500*±0.0	500±0.0	250±0.0
	Roots	-	-	-	-	-	250*±0.0	-	-
T. sericea	Seeds	-	500±0.0	-	500±0.0	-	125*±0.0	62.5±0.0	62.5±0.0
	Twigs	-	500±0.0	-	500±0.0	-	250*±0.0	-	-
L. camara	Seeds	250±0.0	250±0.0	250±0.0	250±0.0	31.25±0.0	31.25*±0.0	62.5±0.0	62.5*±0.0
	Twigs	-	250±0.0	-	-	31.25±0.0	62.5*±0.0	62.5±0.0	125*±0.0
C. tridens	Whole	250±0.0	500±0.0	-	-	125±0.0	125±0.0	62.5±0.0	62.5±0.0
Tetracycline (µg /ml)		2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0

C. E= Clinical *E. coli*; R. E= Reference *E. coli*; C. S= Clinical *Salmonella*; R. S= Reference *Salmonella*; MIC values are expressed in µg/ml; SEM: standard error of the mean; n=3; (-): mean MIC above 500 µg/mL, *p< 0.05 significant between the difference in MICs different parts of the same plant

4.5.4 A comparison of the antibiogram activity of selected antibiotics against clinical and reference bacteria causing gastroenteritis

A higher antibiotic multidrug resistance pattern was observed among all 4 pathogens tested in this study. The reference strain of *E. coli* showed resistance to antibiotics such as amoxicillin 10 µg ciprofloxacin 10 µg, tetracycline 30 µg, erythromycin, penicillin, vancomycin, and ampicillin (Table 6). Among the 10 antibiotics used in the antigram assay, clinical *E. coli* 157:H7 VTEC has shown resistance to 70% with sensitivity only observed in tetracycline (20.33± 0.58 mm), gentamycin (10.00± 0.00 mm), and ciprofloxacin (15±0.00 mm) (Table 6). Meanwhile, the *S. typhurium* reference strain showed resistance to 50% of the standard antibiotics. Furthermore, a 70% resistance was also observed in clinical isolates of *Salmonella* with sensitivity only observed in ciprofloxacin (10±0.00 mm), gentamicin (9±0.00 mm), and tetracycline (18±0.00 mm) (Table 6). Overall, the clinical isolates of *Salmonella* and *E. coli* 157:H7 VTEC showed the highest resistance pattern with activity only observed in 3/10 antibiotics tested in comparison to the reference strains.

In Namibia, morbidity, and mortality due to drug-resistant gram-negative gastrointestinal pathogens are of great concern due to the lack of surveillance data for antibiotics. Moreover, antimicrobials prescription without following the Namibian treatment guidelines for local and regional antimicrobial sensitivity data is also reported in Namibia (Mapiye, 2019). Although antibiotics such as amoxicillin, sulphamethoxazole, ciprofloxacin, and ampicillin are used in Namibia as first-line drugs to treat *E. coli* 157:H7

VTEC and *Salmonella* infection, their uses may not always be effective due to the higher resistance pattern observed in this study. Comparative studies looking at the effectiveness of medicinal plants and antibiotics against both clinical isolates and reference strains are therefore important as they help to eliminate the limitation possessed by the use of only reference strains in drug development. Antibiotic resistance develops as an evolutionary response hence, it has to be visualized as an evolving phenomenon that demands constant surveillance and continuous efforts in identifying the degree of resistance to available antibiotics while creating strategies to combat this problem (Munita Bayer and Arias, 2015; Mohamed *et al.,* 2018). The higher antibiotic resistance pattern against 10 antidiarrheal antibiotics especially against clinical isolate reported in this study is alarming and this makes the plant extracts especially organic extracts of *L. camara, T. sericea, G. tenax,* and *C. tridens* with broad-spectrum and potent inhibitory activity against MDR gastrointestinal pathogens worth further analysis in formulating alternative plant-based medicine for gastroenteritis however the toxicity profile of these extracts have to be determined.

Table 6: Antibiogram activity of standard antibiotics against clinical and reference strains of *Salmonella* spp, *E. coli spp*, Data presented as mean ±SD. *p< 0.05 clinical verse reference strain resistance to antibiotics

Antibiotics (µg/ml)	**Average inhibition of reference strain of *Salmonella* isolates (mm)**	**Average inhibition of clinical *Salmonella* isolates (mm)**	**Average inhibition of reference strain of *E. coli***	**Average inhibition of clinical *E. coli* isolates (mm)**
Amoxicillin 10	9.7±0.58*	0±0.00*	0±0.00	0±0.00
Cephalothin 30	34±1.73*	0±0.00*	18.7±0.58*	0±0.00*
Sulphamethoxazole 25	30.3±0.58*	0±0.00*	20.3±0.58	0±0.00
Ciprofloxacin 10	0±0.00*	10±0.00*	0±0.00*	15±0.00*
Gentamicin 10	28.3±0.58	9±0.00	20.3±0.58*	10.3±0.58*

Tetracyclin 30	25.7±0.58*	18±0.00*	20.7±0.58	20±0.00
Erythromycin	0±0.00	0±0.00	0±0.00	0±0.00
Penicillin 10	20.3±0.58*	±0.00*	0±0.00	0±0.00
Vancomycin 30	0±0.00	0±0.00	0±0.00	0±0.00
Ampicillin 10	0±0.00	0±0.00	0±0.00	0±0.00

4.5.5 Cytotoxicity effect against NIH/3T3cell line

Making assumptions that plant-based extracts are safe due to their long history of use could be misleading and dangerous. Hence, cell-based essays are often used for screening novel formulations to determine if the test molecules are having direct cytotoxic effects. MTT assay was used in the present study. With this assay, viable cells with active metabolism can convert MTT into a purple-colored formazan with a maximum absorbance of 590 nm. When cells die, they lose the ability to convert MTT into formazan; thus, color formation is the marker of only viable cells (Njeru and Muema, 2021).

The cytotoxic activities of the methanol extracts of *L. camara*, *C. tridens*, *G. tenax*, and *T. sericea* were determined at various concentrations on 3T3 cells. Using the absorbance from the microplate reader values of percentage cell viability of the 3T3 cells at different concentrations of the extracts after 48 hours' incubation are shown in Figure 1. The values for cell viability with methanol extracts at the highest concentration of 100µg/mL (Figure 1) were 38.78% for *G. tenax*, 63.08% for *T. sericea*, and 50.52% for *C. tridens*, and 51.68% for *L. camara*. These values were higher than the recorded 29.81% cell viability of the negative control (10% triton x + media). At the lowest concentration of 1.56 µg/mL for all extracts the percentage viability was between 80.05±0.06% and 97.63±0.14%. Gu (2018) also reported minimal cytotoxicity of *T. sericea* to Caco2 cells.

This study is the first to demonstrate the effect of *L. camara*, *C. tridens, G. tenax*, and *T. sericea* methanol extracts on 3T3 cell lines. The results showed that all *L. camara, C. tridens, G. tenax*, and *T. sericea* extracts showed a decrease in cell viability of the 3T3 cell line as the concentration increased. In other studies, a threshold of the cytotoxic concentration (IC_{50}) below 20 µg/mL was considered to be toxic, and above 20 µg/mL to be non-toxic (Mongalo *et al.*, 2017; Afagnigni *et al.*, 2020; Elisha *et al.*, 2017). However, in this study a threshold of IC_{50} 0-20: very toxicity; 21-45: Minimal toxicity; 46-60: Moderate toxicity; 62-100: Not toxic. A study by Koley and Bard, (2010), on the effect of Triton-x on HeLa, has also demonstrated toxicity due to changes in cell morphology, membrane permeability, and viability caused by the presence of Triton-x. Among the four studied extracts, *C. tridens* showed the highest average half-maximal inhibitory concentration (IC_{50}) values of 91.50±0.14 µg/mL after 48 hours. Meanwhile, *T. sericea* extract showed a lower IC_{50} of 65.85±0.07 µg/mL as depicted in Figure 2. However, the IC_{50} of all the extracts is above 20 µg/mL which was set as the threshold for cytotoxicity, the extracts are considered not toxic.

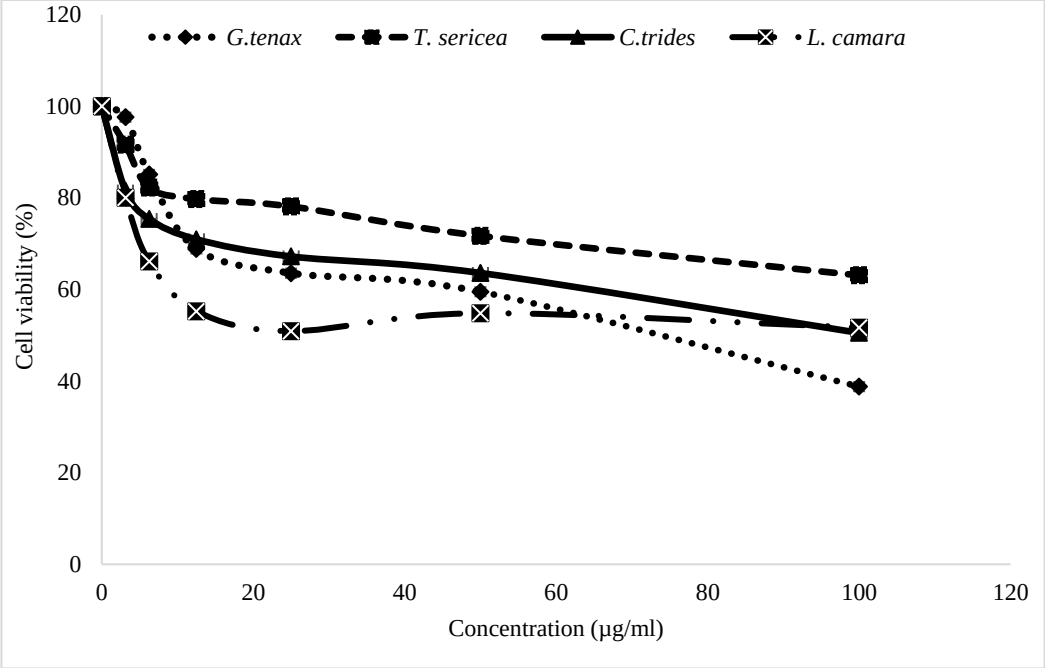

Figure.1. MTT assay on 3T3 cells to evaluate the effect of the *L. camara, C. tridens, G. tenax*, and *T. sericea* methanol extract on the cell growth after 48 hours (X-axis: log concentrations of extracts from 1 to 100 µg/mL) and Y-axis: the percentage of normalized cell viability). Values are the means of three independent experiments.

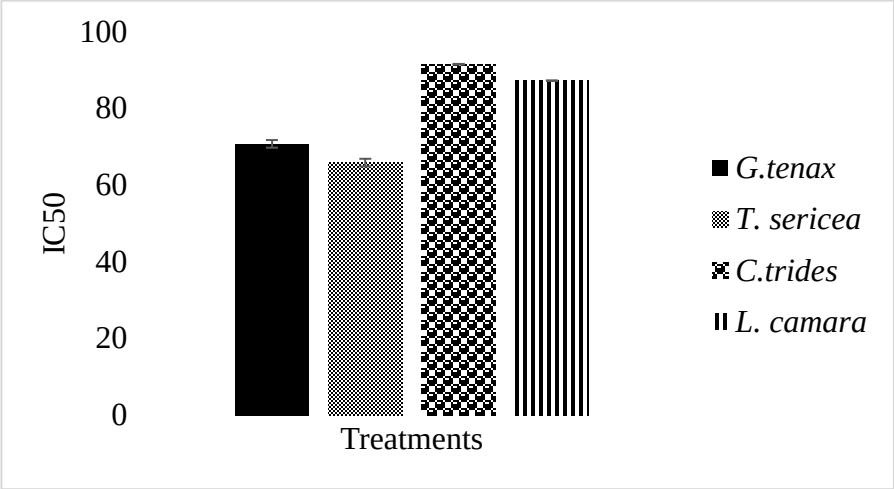

Figure. 2. *In vitro* cytotoxic activity of selected antidiarrheal medicinal plant extracts on 3T3 cell line.

4.6 Conclusion

This study demonstrated that *L. camara, C. tridens*, *G. tenax,* and *T. sericea* antibacterial activity methanol extracts have promising antibacterial activity against multidrug-resistant clinical strains of *Salmonella* and *E. coli*. Moreover, these extracts have proven to be safe in 3T3 cells at concentrations ≤ 100 μg/mL. This makes these extracts potential sources for further complementary and alternative medicine for bacterial gastroenteritis, but only after a safe dosage regimen is validated *in vivo*.

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CHAPTER 5: SYNTHESIS OF CHITOSAN-MODIFIED POLY (LACTIC-CO-GLYCOLIC ACID) NANOPARTICLES WITH A pH-DEPENDENT CONTROLLED-RELEASED KINETICS TO ENHANCE DELIVERY OF POTENTIAL ANTIDIARRHEAL MEDICINAL PLANT EXTRACT TO THE LOWER GASTROINTESTINAL TRACT

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5.1 Abstract

Phytotherapy has been used to treat gastroenteritis in many African countries, with medicinal plant extracts from *Grewia tenax, Corchorus tridens,* and *Lantana camara* showing potent antibacterial properties against gastroenteritis-causing bacteria. However, issues such as uncontrolled metabolism by gastric juices and instability in the gastrointestinal tract due to variable pH of the GI tract, reduce the biotherapeutic properties of these phytomedicines and this has limited their use as alternative/ complementary medicine for gastroenteritis. Hence, there is a need to improve the pharmacokinetic and pharmacodynamic properties of phytomedicine, nanotechnology

utilizes biocompatible nanoparticles to improve these properties, for various medicines. This study aimed at formulating bio-sensitive plant-based chitosan-modified poly (lactic-co-glycolic acid) (CMPLGA) nanoparticles for release in the lower gastrointestinal tract. Nanoparticles were formulated by mixing 12.5 mg/ml of polymers with 120 mg/ml of *G. tenax, C. tridens,* and *L. camara* antibacterial extracts using the modified double (W1/O/W2) emulsion and solvent evaporation method. Formulated nanoparticles were sized and the zeta potential was measured using photon correlation spectroscopy and electrophoretic laser Doppler anemometry. Scanning Electron Microscopy was used to elucidate the nanoparticles' morphology and the encapsulation efficacy was determined using UV-vis spectroscopy. *In vitro* release kinetics of plant extracts from the nanoparticles were determined by sample and separation technique in simulated gastric and intestinal fluid without enzymes. The plant-based CMPLGA nanoparticles prepared were spherical with a size range between 524.4±18.92 and 2582±123 nm, and zeta potential between 2.68±0.08 and 44.2±0.100 mV, plant extract encapsulation efficiency was > 89.8%. The release of phytomedicine from nanoparticles was pH-dependent, with less than 2% extract release at pH 1.2 and over 50% release of the antibacterial extract release at a pH of 7.4. These findings show the CMPLGA nanoparticles could be loaded with antibacterial plant extracts and can protect the phytomedicine from an acidic environment in the upper GI tract. The CMPLGA nanoparticles enhanced the stability of the phytomedicine in acidic conditions similar to those in the upper GI tract and may be a better vehicle for future efficient drug delivery of antibacterial phytomedicine targeting gastrointestinal pathogens in the lower GI tract.

Keywords: Chitosan, Poly (lactic-co-glycolic acid), *G. tenax, T. sericea, C. tridens, L.*

camara, Nanoparticles, *in vitro* release, Encapsulation efficacy

5.2 Introduction

The oral pathway is appropriate for treating intestinal infections due to its ability to offer a transcytosis of the therapeutic compound across the gastrointestinal epithelium, via the lymphatic system through M cells and Peyer's patches (Zhou *et al.,* 2015). This allows controllable delivery, ease of administration, and feasibility for solid formulations. Moreover, in oral delivery, drug molecules trapped within mucus are protected against the shear stresses caused by flowing gastric juices (Homayun, Li, and Choi, 2019).

Despite those advantages, the absorption mechanism of oral drugs is more complex and require the drug to be soluble in different gastric fluid (Ma *et al.,* 2014). Moreover, there are physiological factors within the gut that affect oral drug delivery and absorption of gastrointestinal therapeutic compounds such as gastric emptying rate, intestinal motility, the pH of the gastrointestinal fluids, transit times across the different intestinal segments, intestinal surface area, epithelial permeability, as well as intestinal enzyme and transporter expression (Stillhart *et al.,* 2020). Moreover, due to the harsh conditions along the GI tract delivery vehicles and formulations should be used to gain a stronger delivery with maximum therapeutic efficacy of gastrointestinal therapeutic compounds (D'Souza, 2014; Li *et al.,* 2018).

Medicinal plants in Namibia have shown potential in eliminating multidrug-resistant gastrointestinal pathogens and can play a major role in Phytotherapy and offer a promising source of next-generation medicine for communicable diseases such as diarrhea,

pneumonia, and peptic ulcers (Shatri and Mumbengegwi, 2020, Shatri and Mumbengegwi, 2021; Atanasov *et al.*, 2021). However, similar to antibiotics, phytomedicines are faced with delivery barriers such as breakdown before they reach the targeted site and many other limitations when administered orally as summarized in Figure 1. This necessitated the intake of large doses to achieve a therapeutic dose at the target site, the large doses have the downside of unintended side effects for the treatments.

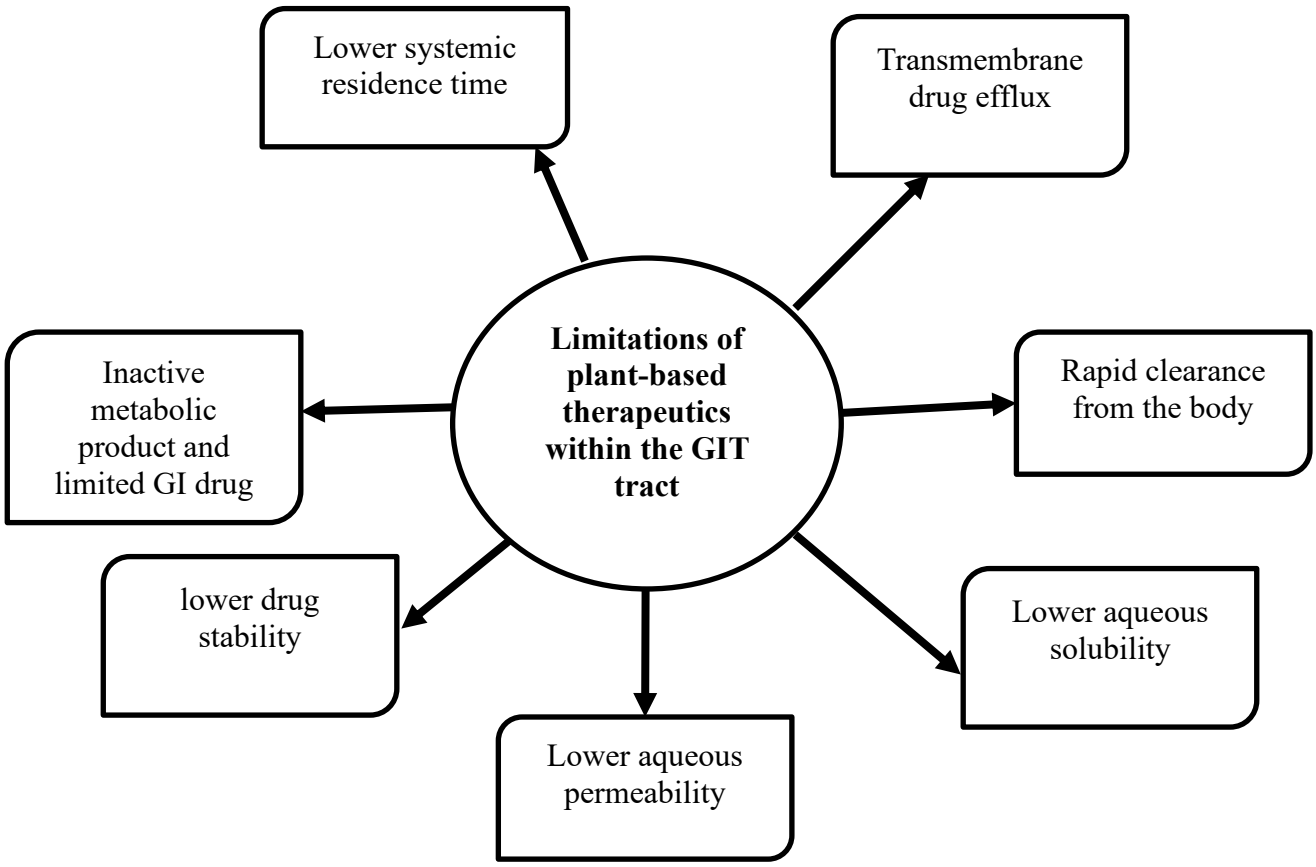

Figure 1: Major limitations of the phytomedicine within the GI tract

Studies have demonstrated superior therapeutic efficacy in using the nano-targeted treatment for *Helicobacter pylori* compared to free antibiotics (Angsantikul *et al.*, 2018). Similarly, the possibility of using bioactive molecules based on polysaccharides, peptides,

proteins, and oligonucleotides to fight off infectious diseases including gastrointestinal conditions can be a breakthrough in treating gastrointestinal conditions such as diarrhea, gastric ulcers, cancer of the gastrointestinal tract, and many gastric disorders if carrier vehicles can be used (Sagadevan *et al.*, 2019).

Among the different drug delivery approaches, nanoparticle-based drug delivery systems have gained considerable attention due to their small size, and strong drug-loading ability. Being nanosized, these structures penetrate the tissues which facilitate easy uptake of the drug by cells, permit an efficient drug delivery, and ensure action at the targeted location. (Yang and Merlin, 2019). Nanoparticles have unique advantages including protecting the drug from premature degrading and interaction with the physiological environment, increasing intracellular penetration, and enhancing drug absorption. A slight change in the physiochemistry of nanoparticles can however significantly impact their interaction with biological pathways and alter the oral bioavailability of drugs.

Several approaches have been considered by modifying the physicochemical properties of the nanoparticles and enhancing the interaction between the nanoparticles and the endocytosis pathways of the gut. This includes controlling nanoparticle size (20 nm to 10 μm), the type of polymer, and the hydrophobicity of the nanoparticle (Ma *et al.*, 2014). Nanoparticles are an effective platform for the delivery of hydrophobic and hydrophilic gastrointestinal therapeutic compounds since the compounds are protected from possible degradation caused by gastric acid and enzymes.

Poly (lactic-co-glycolic acid) (PLGA) is a widely used biodegradable polymer that is approved by Food and Drug Administration (FDA) and European Medicines Agency (EME) as an excipient for parenteral administrations and it is a well-established drug

delivery system in various medical applications. Poly (lactide-co-glycolide) (PLGA) are biodegradable and biocompatible polymers and their microparticles have been used in oral drug delivery systems with a particle size between 500nm to 3000 nm shown to be specifically taken up by M cells. This is achieved by inducing secretory IgA response following the oral delivery of pharmaceutical products (Navarro *et al.,* 2014; O'hagan, 1996). This makes PLGA nanoparticles considerable potential for the development of new and improved pharmaceuticals against a wide range of pathogens, particularly against those which initially infect mucosal sites such as *Salmonella* and *E. coli* species (O'hagan, 1990; Ugboko and De, 2014).

However, one of the major drawbacks of PLGA nanoparticles is that they cannot specifically interact with cells or proteins, which causes an inability to accumulate drugs in target tissues as well as the presence of drug burst release, which could result in side effects (Lu, Lv, Le, 2019; El-Hammadi and Arias, 2022). To overcome these limitations, chitosan can be used to modify the PLGA nanoparticles. Chitosan is a natural cationic polysaccharide with —OH and -NH2 groups it can form covalent bonds with hydrogen. The protonation of an amino group at low pH, which makes chitosan macromolecule charged positively, leads to the mucosal adhesion of chitosan. Moreover, chitosan has higher biocompatibility and biodegradability rate. The use of polymers such as PLGA particles grafted with gelatin or chitosan has shown sufficient mucoadhesion to Peyer's patches, which is significant for nanoparticle uptake by Peyer's patches (Feczkó, 2021). Because the pH of each region of the human body is different chitosan delivery system can achieve targeted delivery of drugs (Bhadra, Prajapati, and Bhadra, 2016). The protonation observed when using chitosan as an enteric coating for nanoparticles offers

protection of the drug from the strong acid pH of the stomach making it bioavailable at another pH. This results in the quick release and high drug concentration gradient, which is important for drug absorption (Bhadra *et al*., 2016).

This study was conducted to develop CMPLGA nanoparticles for drug delivery of *G. tenax, C. tridens,* and *L. camara* derived phytomedicines with antibacterial properties against multidrug-resistant gastroenteritis-causing clinical bacteria. The medicinal plants were selected and collected based on their ethnomedicinal uses by the Awambo people in northern Namibia as described by Shatri and Mumbengegwi, (2022) (Chapter 3 of this dissertation). The selected plants have also shown potent potent antibacterial activity with MIC below 100 µg/ml Multidrug resistant clinical isolates of *E. coli* and *Salmonella* spp as shown in chapter 4. It was hypothesized that the use of Chitosan and PLGA nano-systems will improve the drug delivery properties of phytomedicines over that of the free phytomedicines.

5.3 Materials and Methods

5.3.1 Materials

Terminalia sericea seeds, *Lantana camara* seeds and twigs, *Grewia tenax* roots, and *C. tides* whole plant (5 kg) were collected from Iikokola village in the Omusati region and were dried at room temperature, in the dark, for 4 weeks. Poly (vinyl alcohol) (PVA) (Mw = 13000-23000 kDa, S% = 87-89%, hydrolyzed was provided by Qingdao Lantai Pharmaceutical Co. Ltd., Qingdao, China, Poly (lactic-co-glycolic acid) (PLGA 50/50, the average molecular weight, Mw, 70 kDa) was purchased from the Institute of Medical Devices (Shandong, China). Chitosan (lower molecular weight) was purchased from

Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

5.3.2. Preparation of Extracts

T. sericea seeds, *L. camara* seeds, and twigs, *G. tenax* roots, and *C. tides* whole plant materials were collected from the Omusati region in April 2017 with a plant collection permit issued by the National Commission on Research Science and Technology (NCRST) and the Ministry of Environmental and Tourism (permit number 2221/2017), in compliance with the national Access Benefit Sharing (ABS) laws. The collected plant materials were washed, shade dried, and ground into powders. The powders were macerated in methanol (2 g/ml) for 48 hours on a shaker in the dark at room temperature. The plant material was filtered using Whatman no 1 filter paper and concentrated under reduced pressure using a rotary evaporator and a freeze dryer. The residual extracts were collected and kept at 4 ºC, in capped vials until required to prepare nanoparticles (Shatri and Mumbengegwi, 2020).

5.3.3 Preparation of chitosan-coated poly (lactic-co-glycolic acid) nanoparticles containing plant extracts

Nanoparticles containing antibacterial phytomedicine were prepared using a modified double ($W_1/O/W_2$) emulsion and solvent evaporation method (Arafa *et al.,* 2020) as depicted in Figure 2. Briefly, 120 mg/ml plant extract in methanol was vortexed for 3 minutes. Under stirring 2 % Poly (vinyl alcohol) (PVA) 2% was added to the mixture to form the internal water phase W_1. Exactly 12.5 mg/ml poly (lactic-co-glycolic acid (PLGA) in Dichloromethane (DCM) under stirring was used to prepare the oil phase (O). The internal water phase was added drop by drop into the oil content forming the W_1/O primary emulsion. The primary emulsion was homogenized using a High-Speed

Homogenizer, 1.8 Kg., D-500 Pro at 10 000 rpm for 5 minutes on ice. A W_2 phase was formed using 2.5 ml of 0.1% chitosan and 10 ml of 2% Poly Vinyl Alcohol (PVA) as a stabilizing agent. The primary emulsion was added to the external water phase and homogenized at 10 000 rpm for 5 minutes on ice to form a (W_1/O/W_2) double emulsion. The double emulsion was left on the stir plate for 24 hours at 25 °C to allow DCM and methanol to evaporate. The flasks with the emulsion were covered with Parra film and left to stir for another 24 hours. The emulsions were centrifuged at 121968 xg for 15 minutes at 19.2 °C. The CMPLGA pellets were added to cryoprotectant-trehalose, stirred until fully mixed, and analyzed for particle size and zeta potential, and polydispersity index. The remaining plant-based CMPLGA pellet emulsions were added to conical flasks and initially frozen with liquid nitrogen before putting them on a freeze drier for 72 hours for further drying into powder.

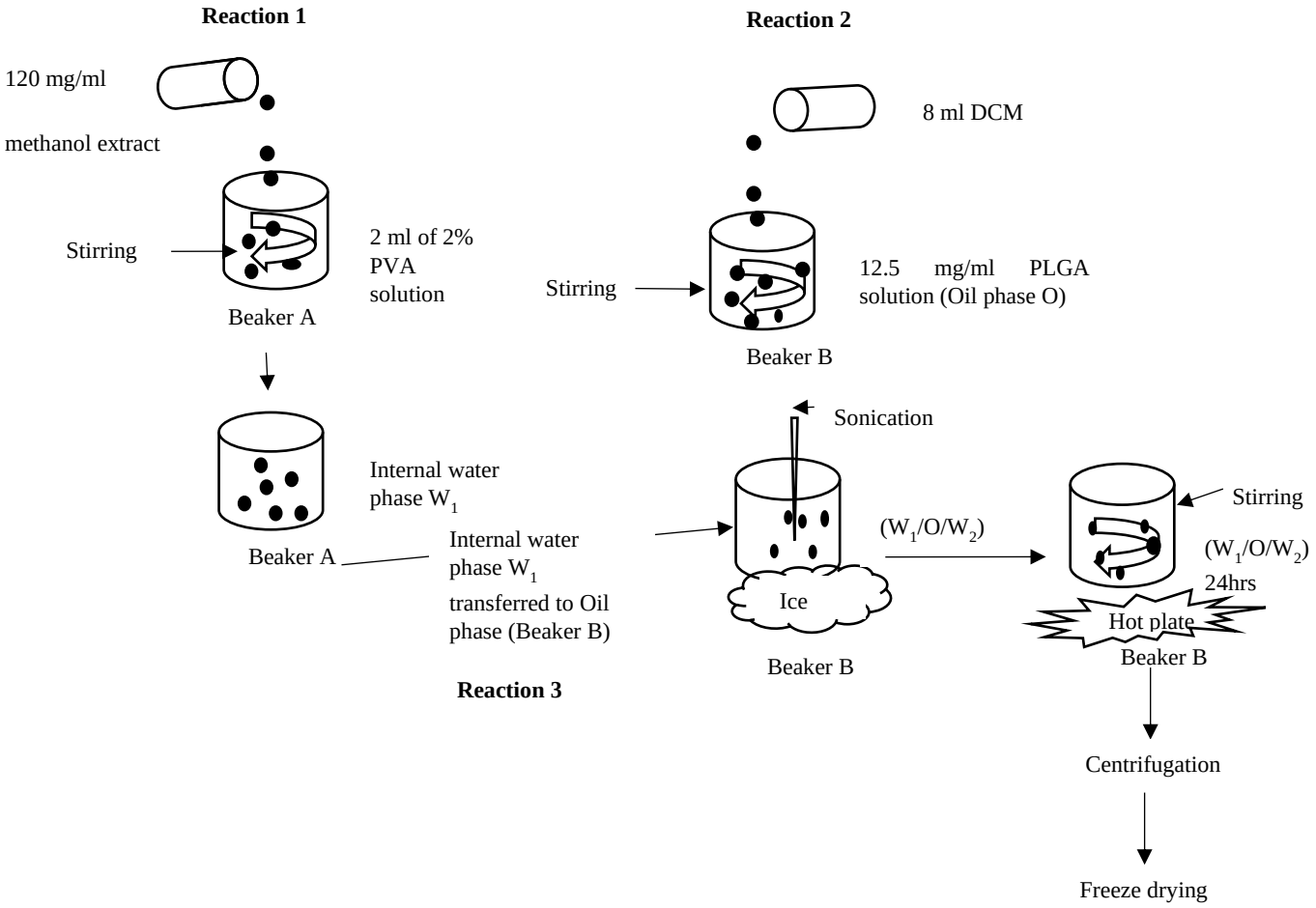

Figure 2: Schematic diagram of medicated nanoparticle preparations (Shatri, 2022).

5.3.4 UV- Vis analysis of Nanoparticles

UV-visible spectra were recorded using a Shimadzu UV-visible 1800 spectrophotometer for the confirmation of nanoparticle formation. Briefly, the nanoparticle emulsions were diluted in distilled water (1:1) and vortexed for 5 minutes. The diluted nanoparticle samples were analyzed using a UV-Vis spectrophotometer in the wavelength range of 200–700 nm. The experiment was done in experimental triplicates experimentally.

5.3.5 Measurement of the nanoparticle size and zeta potential

Particle size and zeta potential of double emulsions of nanoparticle suspension were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zeta-sizer (Malvern Instruments Ltd., Worcestershire, UK). Briefly, the nanoparticle double emulsions sample was diluted in deionized water (1:1) and vortexed for 5 minutes. The solutions were transferred to cuvettes and analyzed for zeta potential and nanoparticle size and polydispersity index using a Zeta-sizer at a fixed angle of 90° at 25 °C in experimental triplicates.

5.3.6 Observation of the morphology by Scanning Electron Microscopy

The morphology of nanoparticles was carried out by Scanning electron microscopy (SEM) (Jeol JEM1200EX, Tokyo, Japan). Approximately 1 mg of freeze-dried plant-based CMPLGA nanoparticles was attached to a carbon tape and firmly attached to the slide by pressing the carbon tape with the sample on the metal slide. The samples on the slides were coated with the Quorum carbon coating machine for 5 minutes. The samples were analyzed for particle size and morphology at 3000x and 30000x magnifications.

5.3.7 Determination of the encapsulation efficiency

Exactly, 0.2 mg/ml of each of the 6 formulated plant-based CMPLGA nanoparticles in dichloromethane was vortexed for 5 minutes. Exactly 1 ml of methanol was added to each mixture and the solutions were vortexed for 5 minutes. The CMPLGA nanoparticle solutions were stored at -4°C overnight to enable the nanoparticles to fully dissolve. Centrifugation of the CMPLGA solutions was done at 1008 xg for 10 minutes and the supernatants were collected. The amount of drug within the supernatant was determined

using a UV- spectrophotometer by measuring the absorbance of 1 ml of the supernatant solutions at 250 nm. The concentration of the extract was calculated using the calibration curves shown in Appendix H. The experiment was repeated independently 3 times.

$$EE(\%)= \frac{\text{The total amount of extract(mg)} - \text{Free amount of extract in the supernatant(mg)}}{\text{The total initial amount of extract (mg)}} \text{ x } 100$$

$$\text{The yield of Nanoparticles (\%)} = \frac{\text{Weight of Nanoparticles}}{\text{Total weight of polymer and extract}} \text{ x } 100$$

5.3.8 *In vitro* pH-controlled release of phytomedicines in simulated gastric fluid, and simulated intestinal fluid without pepsin and pancreatin solutions

In vitro release of plant-based CMPLGA (G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), and CMPLGA(LT), nanoparticles were done by sample and separation technique in simulated gastric fluid (1.2 pH) and the intestinal fluid (7.4 pH) using UV-vis spectroscopy with slight modification. The chemical compositions of the simulated gastric and intestinal fluids are in Appendix K. Free plant extract concentrations were also monitored over 24 hours at 1.2 and 72 hours for nanoparticles. Experiments were carried out by suspending 10 mg of the nanoparticles in Elmer flasks containing 175 mL of the release media. The Julgbo SW22 shake incubator was set at 37±0.5◦C and 120 rpm. At predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 24, 48, and 72 hours), 5 ml of the release medium was withdrawn. The collected aliquots were filtered and centrifuged at 16128 RCF for 20 minutes. The supernatants were removed after every centrifugation and the precipitated nanoparticles were re-suspended in 5 ml of fresh media and were then put back in the Elmer flasks on the shaking incubator and spectrophotometrically analyzed for plant extract concentration at 250 nm using a Uv spectrophotometer: JASCO. The blank was the CMPLGA(EE) nanoparticles that were treated the same as the medicated.

The amount of extract released in supernatants was determined from the calibration curve used for the encapsulation efficacy calculation and all equations used in the calculation are shown in Appendix H. The experiment was repeated 3 times independently (Kesente *et al.,* 2017).

5.4 Statistical analysis

All values are expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA (using Dunnett's post-test), and $p < 0.05$ was considered statistically significant. All experiments were repeated 3 times either independent or experimental replicates as shown under every method.

5.5 Results and Discussion

5.5.1 UV-Visible analysis of Nanoparticles

UV–vis spectroscopy is the method widely used to confirm the successful synthesis of Chitosan-modified PLGA nanoparticles by monitoring the correlation between the peak observed at a given wavelength and the average nanoparticle diameter (de Melo *et al.,* 2020). In this study plant-based CMPLGA nanoparticles: (CMPLGA(G) containing *G. tenax* extract, CMPLGA(T) containing *T. sericea* extract, CMPLGA(C) containing *C. tridens* extract, CMPLGA(LS) containing *L. camara* seeds extract, and CMPLGA(LT) containing *L. camara* twigs extract, CMPLGA(EE) as an empty encapsulation were analyzed. In this study, the optical properties of nanoparticles were analyzed by UV-Visible spectroscopy and are shown in Figure 3 A-E. A UV-Visible spectrum of empty nanoparticles (CMPLGA(E) obtained broad absorption band spectra compared with plant-based PLGA nanoparticles (sharp intensity). Moreover, the absorption peak

wavelengths were at 230, 245, and 260 nm in the UV region in all plant-based CMPLGA nanoparticles and the empty nanoparticles encapsulation which was due to the formation of nanoparticles. But higher intensity distinct peak levels (of 275 and 250 nm in CMPLGA (LS)), (240 nm in CMPLGA(LT)), (265 nm in CMPLGA(G), and (250, 255 nm CMPLGA)) were also observed. Other studies have also shown that plant-based PLGA nanoparticles form peaks between 227 and 280 nm (Kumari, Adhikari, and Yadav, 2012).

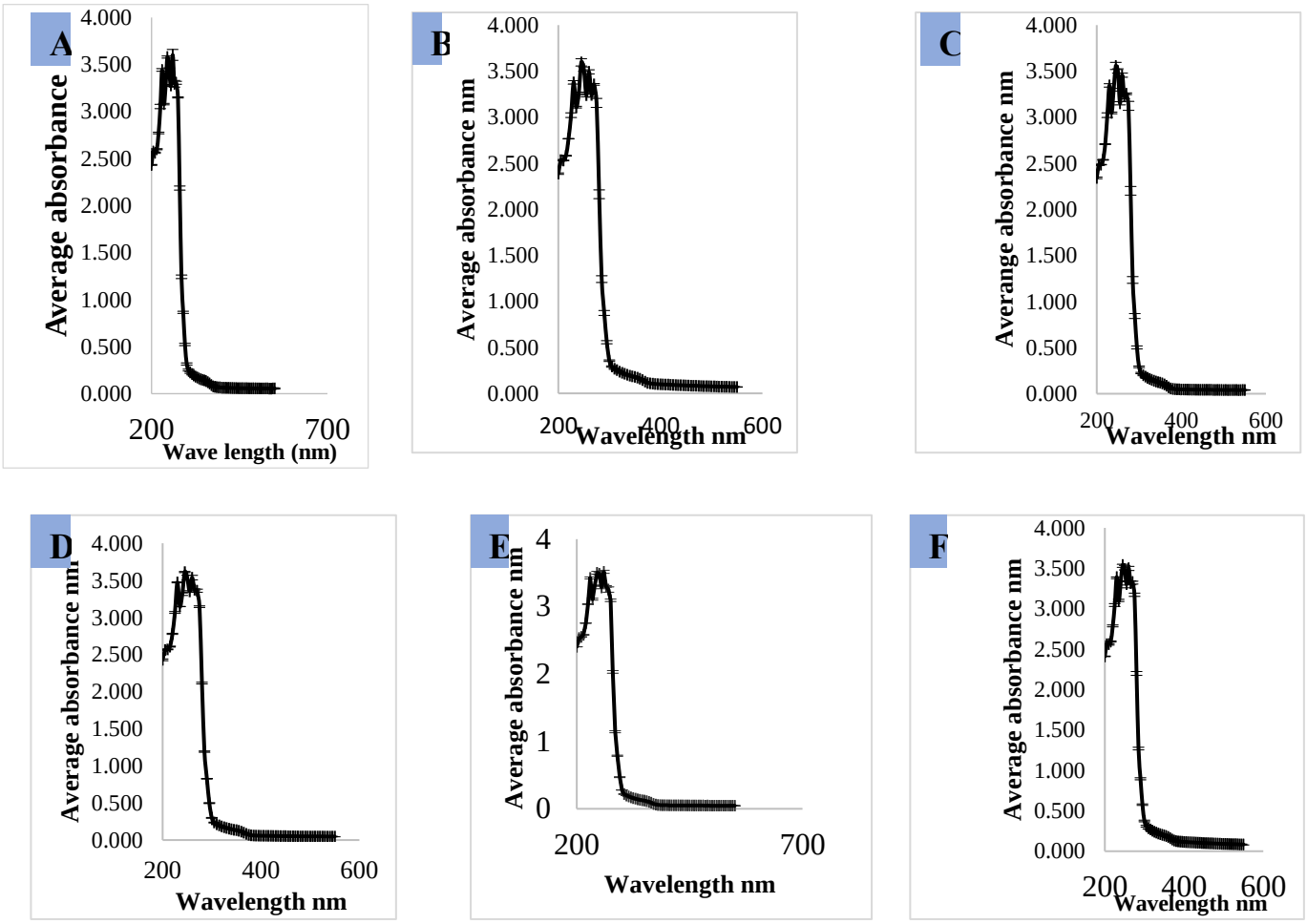

Figure 3: UV-visible spectrum of synthesized nanoparticles of A: CMPLGA(T), B: CMPLGA(LS), C: CMPLGA(LT), D: CMPLGA(G), E: CMPLGA(C), and F: CMPLGA(EE)

5.5.2 Characterization of CMPLGA nanoparticles

The present study is the first to encapsulate phytomedicine in CMPLGA nanoparticles. The enteric nanoparticles were prepared using the method of double (W1/O/W2) emulsion and solvent evaporation. The nanoparticle suspension was oyster white in color and translucent with visible sky-blue opalescence. The morphological characteristics of the enteric nanoparticles were observed using SEM (Figures 4 a and b), and the nanoparticles were spherical, and uniform in shape. Figure 4 shows the morphology of CMPLGA(T) nanoparticles, the morphology of all the other nanoparticles is in Appendix J. The average sizes of CMPLGA nanoparticles are summarised in Table 1. Among the formulations, CMPLGA(LS) showed a small average size of 524.4±18.92, while CMPLGA(T) showed the largest nanoparticles' average size of 2582±123.3. These were compared to the CMPLGA(EE) nanoparticles which showed an average size of 233.6± 168.8 nm (Table 1). The size of the formulated nanoparticles in this study is within the nano-range (20 nm to 10 µm), reported in other studies to have shown enhanced interaction between the nanoparticles and the endocytosis pathways of the gut (Ma *et al.*, 2014).

The polydispersity index (Pdi) was the measure of the distribution of nanoparticles within the sample and the results are summarized in Table 1. Of all the CMPLGA nanoparticles CMPLGA(LS) showed the lowest Pdi of 0.435±0.036 while CMPLGA(T) and CMPLGA (C) showed the highest Pdi of 1.00±0.00. These were compared to the CMPLGA(EE) nanoparticles which showed an average Pdi of 0.439±0.100. A Pdi value of 0.1- 0.3 indicates a narrow size distribution, (monodisperse), and a value >0.3 indicated a very broad size distribution (polydisperse) (Li *et al.*, 2018). Hence the nanoparticles developed have a broad particle size distribution (Appendix J). The zeta potential of nanoparticles is

linked to the stability of the nanoparticles, and it has an important effect on the storage stability of the colloid dispersion system (Tantra *et al.,* 2010). Factors that govern the uptake of particles from the gastrointestinal tract are particle size, surface charge, and physicochemical properties of the particle. It is important to understand that the positively charged drug-loaded nanoparticles are expected to interact with negatively charged sialic acid and fructose residues of mucin in the intestine by electrostatic interactions which are necessary for effective drug uptake (Honary and Zahir, 2013).

The results of the zeta potential of CMPLGA are summarised in Table 1 where CMPLGA(T) showed the lowest zeta potential of 2.68±0.08, while CMPLGA(LT) showed a higher zeta potential of 44.2±0.100. Other studies have shown that a positive zeta potential shows stability in nanoparticle formulated. Nanoparticle surface is a very important factor to consider in targeting drug delivery hence, ensuring that the zeta potential of the nanoparticles is within range helps to offer an effective release profile of the plant extracts as well as their circulation in the bloodstream and absorption into the body membranes (Ardawsari *et al.,* 2020). Pictures of zeta potential, size, and Pdi are shown in Appendix J.

Table 1: CMPLGA nanoparticles' size, zeta potential, and polydispersity index

Nanoparticles	Size (nm)	Zeta potential (mV)	Pdi
CMPLGA(G)	605.5±29.89	17.1±0.289	0.605±0.014
CMPLGA(T)	2582±123.3	2.68±0.08	1.00±0.00
CMPLGA(C)	5734±1225	32.8±0.451	1.00±0.00
CMPLGA(LS)	524.4±18.92	23.30±0.208	0.435±0.036
CMPLGA(LT)	722.6±16.92	44.2±0.100	0.467±0.022
CMPLGA(EE)	574.6±0.100	36.8±0.08	0.439±0.100

Results presented as mean ± Standard deviation, n=3, CMPLGA: Chitosan-modified PLGA.

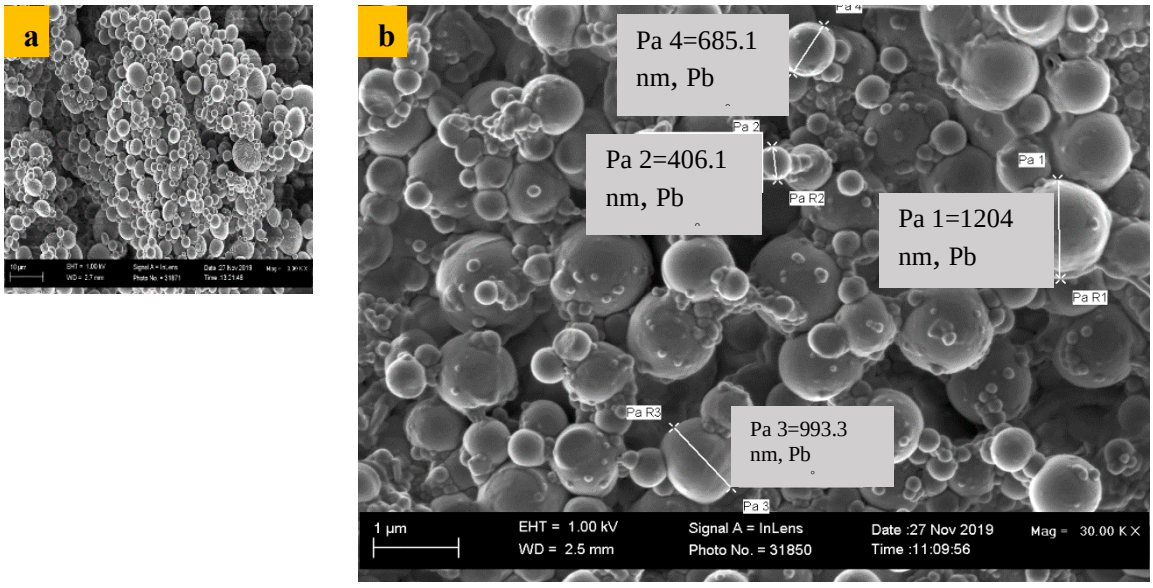

Figure 4: Scanning Electron Microscopy (SEM) image of plant-based CMPLGA(T) nanoparticles (a) Particle morphology at 3000x magnification, (b) Particle morphology at 30000x magnification (Pa= Particle diameter and Pb= particle degree.

5.5.3 Determination of drug encapsulation efficiency

The encapsulation efficiency of enteric PSS-NP was calculated using equations in Appendix H. The mean encapsulation efficiency of different nanoparticles was: CMPLGA(LS) 89.80%, CMPLGA(LT) 95.8 %, CMPLGA(T) 97.4 %, CMPLGA(C) 95.3%, and CMPLGA(G) 95.6 %. Considering the physicochemical properties of macromolecular drugs and soluble polysaccharides, the values were normal. Studies have linked the increase in homogenization cycles to higher EE%. Moreover, the use of chitosan in formulating chitosan-coated PLGA nanoparticles has also been linked to higher EE% for CMPLGA nanoparticles (Arafa, *et al.,* 2020; Sampathkumar *et al.,* 2019).

5.5.4 *In vitro* pH-controlled release kinetic studies

The main objective of this chapter was to prepare nanoparticles that are suitable and can remain intact at lower stomach acidic pH and that have a suitable release profile at the ileum pH of 7.4. The *in vitro* release experiments were conducted at 1.2 and 7.4 pH at 37±0.5 ◦C. These pH values were selected based on the standard pH values for the stomach and ileum respectively. The release profile is depicted in Figures 5 A-E. So, the prepared nanoparticles were suspended in different simulations to determine their stability. As shown in (Figures 5 A-E), the *in vitro* release profiles of plant extracts in the simulated gastric and intestinal fluid of pH 1.2 and 7.4, respectively for 0–72 hours to mimic the physiological conditions in the living organisms.

The release of all the nanoparticles was pH-dependent. The release of extracts from nanoparticles was retarded at pH 1.2 (<2 %) as there was an insignificant extract release at acidic pH 1.2 (Figure. 5 A-E). There was a decrease of over 87.8% in levels of plant extracts in simulated fluids within 2 hours when non-encapsulated, this has alluded to instability at low pH. There was a controlled release at 7.4 pH. This indicates the protection of a significant amount of the extract encapsulated in the nanoparticle from the acidic environment of the stomach by the polymers. However, all nanoparticles showed a significant burst release of extract (≥13.4%), and CMPLGA(LS) showed the highest burst release of extract of 78.6 % at 2 hours in the simulated intestinal fluid at pH 7.4 (Figure 5 E) which was due to the unencapsulated plant extract. After that, extracts "escaped" at a constant maximum rate from the nanoparticles but never reached a 100% cumulative release. The release profile in the simulated intestinal fluid indicated a possible release of the extract in the intestinal fluid, where uptake and absorption of the extract are expected to take place by the intestinal cells. Statistically, there is a significant difference in the release properties of extracts and nanoparticles at the pH of 1.2 and 7.4 with $p<0.001$.

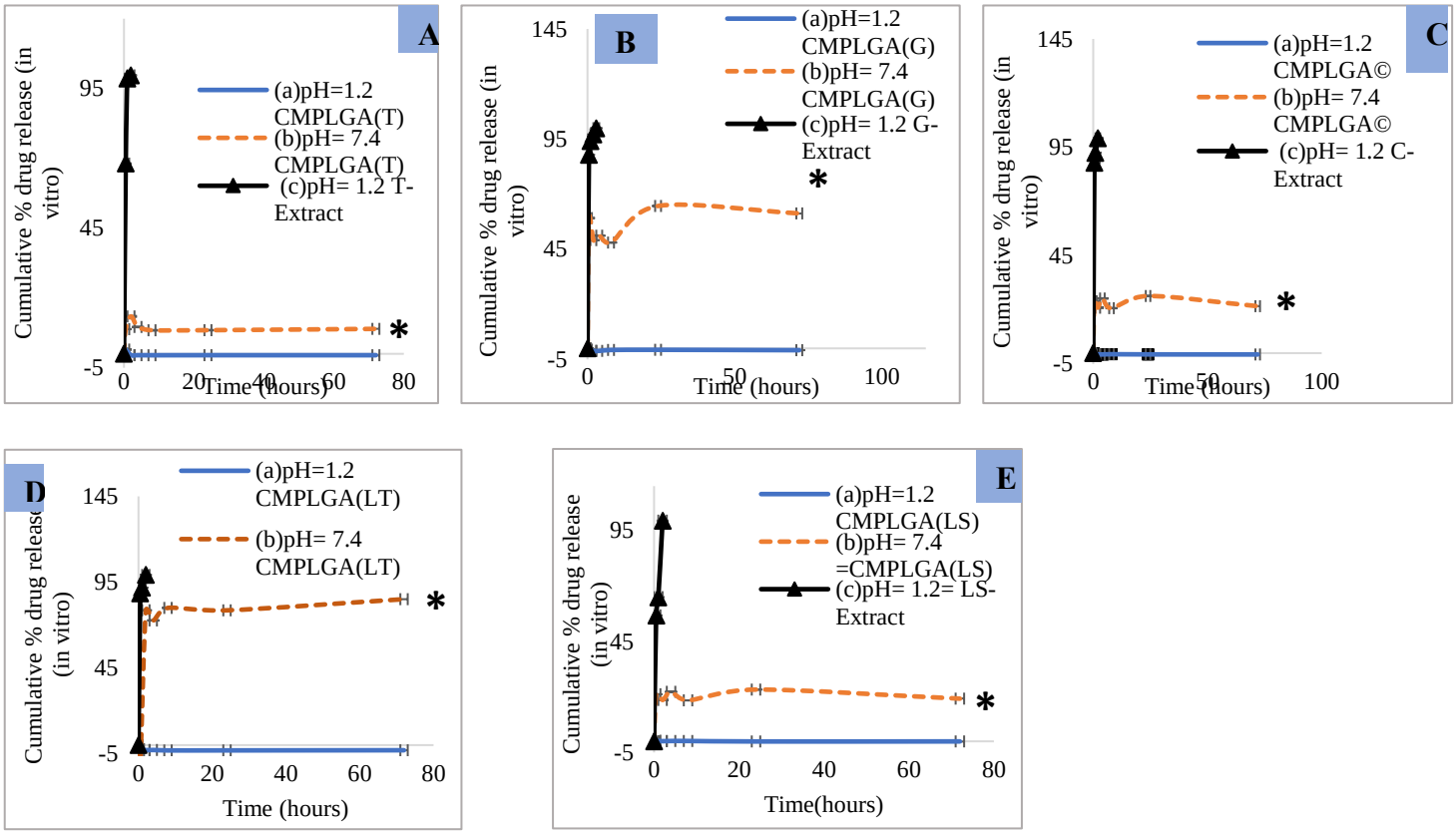

Figure 5: Release kinetics of A: CMPLGA(T), B: CMPLGA(G), C: CMPLGA(C), D: CMPLGA(LT), and E: CMPLGA(LS) dispersed in simulated gastric fluid pH 1.2 and simulated intestinal fluid pH 7.4, (n =3, Mean ± SD), $p<0.001$ for comparison of release in simulated gastric and intestinal fluids

This is the first-time development of novel CMPLGA nanoparticles to formulate pH-dependent nanoparticles for the delivery of phytomedicine for gastroenteritis-causing pathogens. There was a significant difference ($p > 0.05$) in the release profiles of the various formulation. Studies have displayed that the release of the drug depends largely on the concentration of the drug-loaded and the polymer used for encapsulation (Son, Lee, and Cho, 2017). Adding chitosan to nanoparticles theoretically slow the release of the extracts by increasing the path length through which the drug must diffuse (Mumuni *et*

al., 2019). Nanoparticles loaded with CMPLGA(LT) showed a significant and sustained release profile compared to other nanoparticles.

Salmonella Typhi and Enterogenic *E. coli* are common human gastroenteritis pathogens that reside in the M-cells of the Peyer patches (Ugboko and Nandita, 2014). This is the same rote required for most drug absorption into the lymphatic system. Hence being able to develop a nanoparticle that can target *Salmonella* and Enterogenic *E. coli* within the M- cells and protect it from acid and enzymatic degradation can offer an effective target-specific solution to gastroenteritis. This will require being able to maintain the nanoparticles within the M-cells for a while (by increasing particle size) to increase efficacy and using factors such as pH, and surface charges to control the release of the therapeutic from the nanoparticles (Sundar and Prajapati, 2012).

The lower extract release observed in the simulated gastric fluid (pH 1.2) is consistent with the report of similar work where the use of the cationic group on thiolated trimethyl chitosan showed a decrease in the release of the drug from nanoparticles (Lima *et al.,* 2018; Prabaharan and Mano, 2004). Chitosan forms colloidal particles and entraps bioactive molecules through many mechanisms, including chemical crosslinking, ionic crosslinking, and ionic complexation. A possible alternative of chitosan by chemical modification also has been useful for the association of bioactive molecules to polymers and for controlling the drug release profile (Prabaharan and Mano, 2004; Mikušová and Mikuš, (2021). This release may be due to the plant extracts adsorbed on the surface of the nanoparticle, which is rapidly released due to weak interactions between the drug and polymer, characterizing a burst effect.

Although there was a burst release observed in all nanoparticles at the pH of 7.4, a burst release was not observed at 1.2 pH in the formulations, and this could be due to the use of chitosan which is known to offer a decreased burst effect in encapsulated drug release. Moreover, using chitosan in a formulation also offers other benefits such as increased stability of macromolecules such as proteins; enhancement of the zeta potential inversion, promoting cellular adhesion, and retention of the delivery system at the target site (Moraru *et al.,* 2020). Chitosan also helps in the pH-dependent release of drugs by playing a dual function in protecting the pharmaceutical product against the action of the enzymes and gastric fluids, and reduction of the gastrointestinal (Khanal *et al.*,2016). While drug bust release always occurs during *in vitro* release, due to drug absorption on the surface of the nanoparticles, studies have also shown that a strong burst release should be avoided, since it can reduce the effectiveness of the drug and may cause side effects on the human body (Rodrigues de Azevedo *et al.,* 2017). It has also been proven that around 60% of the drug is released in the first 24 hours from PLGA followed by slow release over days as observed in most of the formulations in this study (Afara *et al.,* 2020).

Rodrigues de Azevedo *et al.,* (2017) have shown that the amount of drug released during a burst is mostly influenced by the formulation characteristics and the synthesis parameters, whereas the drug release kinetics is also influenced by the molecular properties of the drug. The release of plant extracts in different simulated body fluids showed that the pH of the simulation has a direct effect on the release profile of the extract. CMPLGA nanoparticles prepared with *T. sericea*, *L. camara*, *G. tenax,* and *C. tridens* have the potential for oral administration since they were efficient in retaining most of the extract contents under acid conditions. Thus, CMPLGA nanoparticles may be absorbed

by the intestinal cells, leading to improved extract bioavailability (Lima *et al.*, 2018). The use of increased epithelial contact time thereby increased absorption of the entrapped drug (Navarro *et al*., 2014). The sustained release behavior observed in these CMPLGA nanoparticles is attributed to the diffusion of the encapsulated plant extracts from the polymeric matrix after the erosion and hydrolytic cleavage of PLGA. Furthermore, the chitosan coating the PLGA nanoparticles' surface plays a vital role in achieving a prolonged release behavior (Azzazy *et al.*, 2021), which in return increases the healing effect of plant extracts by prolonging the contact time with gastrointestinal epithelial cells.

5.6 Conclusion

These findings demonstrate that nanoparticles do not only protect the payload of phytomedicines from an acidic environment of the stomach but also exhibit a controlled release of plant extracts in the intestinal fluid where uptake and absorption of the extract by the intestinal cells occurs. The nanoparticles formulated in this study have the advantage of sustained drug release and could be used as carriers of antidiarrheal phytomedicine. However, the toxicity and efficacy of the nanoparticles should be determined.

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CHAPTER 6: THE EFFECT OF LYOPHILISATION ON THE PHYSIOCHEMICAL AND BIOLOGICAL PROPERTIES OF PLANT-BASED CHITOSAN MODIFIED- PLGA NANOPARTICLES

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6.1 Abstract

Medicinal plant extracts from *Terminalia sericea, Lantana camara, Grewia tenax,* and *Corchorus tridens* have shown potential as alternative and complementary medicine for gastroenteritis due to their broad-spectrum antibacterial activity against diarrheal bacteria. However, barriers such as poor uptake and metabolism by gastric acid reduce the antibacterial activity of plant-based medicine, and this limits their mainstream uses. Encapsulating plant extracts into FDA-approved polymers such as poly (lactic-co-glycolic acid) and chitosan have shown benefits such as improved antibacterial and cytotoxicity activity as well as better bioavailability, and mucoadhesive properties. However, since nanoparticles in a suspension are prone to swelling and bursting, lyophilization of nanoparticle suspensions can be a promising technology to improve nanoparticle stability during long-term solid storage. The main goal of this study was to

investigate the effect of lyophilization on the physicochemical and biological properties of plant-based Chitosan-Modified nanoparticles (CMPLGA) stored at different temperatures over time. The Plant-based CMPLGA nanoparticles used were formulated using chitosan, PLGA, and antibacterial plant extracts from *Grewia tenax*, *Terminalia sericea*, *Corchorus tridens*, and *Lantana camara*. The formulated nanoparticles were lyophilized and analyzed for size, polydispersity index, zeta potential, and pH. The antibacterial and cytotoxicity of the lyophilized nanoparticles were also determined by agar disc diffusion and MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assays respectively. There was a significant increase in nanoparticle size after lyophilization due to aggregation with a greater difference in the size of 3666.5± 250 nm observed in CMPLGA nanoparticles containing *L. camara*. Moreover, all the plant-based CMPLGA nanoparticles showed an insignificant change in the zeta potential ≤ 7 mV) and polydispersity index of ≤ 0.6 after lyophilization, except for the empty CMPLGA nanoparticles. The antibacterial $_{activity}$ of the nano dissolution was between 18.33±0.009 and 12±0.009 even between 4-24 weeks of storage at 4 °C against both *Salmonella* and Verotoxigenic *Escherichia coli* and the lowest Minimum inhibitory concentrations of 6.25±0.0 and 62.25 µg/ml respectively. Moreover, the antibacterial activity of the intact-nano solution stored at 4 and 25°C did not show a significant increase in antibacterial activity with time indicating only minimal nanoparticle swelling and leakage. While cytotoxicity of the nanoparticles is normally associated with prolonged storage, the IC_{50} of the formulated nanoparticles ranged between 66.7±0.005 µg/ml in CMPLGA containing *L. camara* seed extract and 76.6±0.009 µg/ml in CMPLGA containing *C. tridens*. This was comparable to the empty CMPLGA formulation which showed an IC_{50}

of 88.1±0.007 µg/ml. The IC_{50} was higher than the 50 µg/ml that was set as a threshold for nanotoxicity and this indicates that the nanoparticles are not significantly toxic to the NIH/3T3 mouse embryonic cell line. Hence, the use of CMPLGA nanoparticles enhanced the antibacterial activity and showed minimal cytotoxicity of *Terminalia sericea, Lantana camara, Grewia tenax* roots, and *Corchorus tridens*.

Keywords: Chitosan, Poly (lactic-co-glycolic acid), *G. tenax, T. sericea, C. tridens, L. camara*, Nanoparticles, Cytotoxicity, Lyophilization

6.2 Introduction

About, 50% of the major pharmaceutical compounds and their derivatives available today are obtained from natural resources such as medicinal plants (Nie *et al.,* 2020). Plant-based pharmaceutical compounds possess unique advantages, such as lower toxicity and side effects, low price, and good therapeutic potential. Studies conducted in Namibia have proven that medicinal plants such as *Grewia tenax, Lantana camara, Terminalia sericea,* and *Corchorus tridens* are packed with bioactive compounds such as alkaloids, saponins, flavonoids, coumarins, anthraquinones and tannins (Mapiye, 2019; Iikasha *et al.,* 2020; Shatri and Mumbengegwi, 2020). Moreover, these plant extracts have also shown potent antibacterial activity and have proven to be non-toxic to NIH/3T3 fibroblast cell line that was isolated from a mouse NIH/Swiss embryo (Shatri and Mumbengegwi, 2022). However, like other orally administered antidiarrheal agents and antibiotics, concerns regarding poor bioavailability, and poor solubility, poor absorption in the gastrointestinal tract, issues with target-specific delivery. biocompatibility and uptake present a greater challenge in using them as medicine, which limits their chances of making it to clinical trial phases (Anand *et al.,* 2019).

Nano-delivery systems are a relatively new but rapidly developing science where materials in the nanoscale range are employed to serve as means to deliver therapeutic agents to specifically targeted sites in a controlled manner (Patra *et al.,* 2018). Studies have shown that using FDA-approved polymers such as poly (D, L-Lactide-co-glycolide) (PLGA) and chitosan as carriers for phytomedicine serves numerous benefits such as increased gastrointestinal tract transit time of nanoparticles and consequently bioavailability of entrapped active components due to the positive charge and mucoadhesive properties of natural polymers such as chitosan (Pathomthongtaweechai and Muanprasat, 2021). Combining chitosan and PLGA in a formulation can form Chitosan-Modified PLGA (CMPLGA) nanoparticles that can be useful and can utilize the mucoadhesive property of chitosan and PLGA's ability to powerfully entrap hydrophobic and hydrophilic drugs (Mohammed *et al.,* 2017). Various formulations of CMPLGA nanoparticles have been applied to promote sustained drug release, for drug targeting, and to increase drug absorption (Lu *et al.,* 2019; Sharifi-Rad *et al.,* 2021).

The suitability of using CMPLGA nanoparticles as carries for phytomedicine within biological systems is well documented due to their high bio-absorbability (Antunes *et al.,* 2021; Zhang *et al.,* 2016; Rezvantalab *et al.,* 2018; Casadidio *et al.,* 2019). Therefore, using CMPLGA drug delivery systems for phytomedicine within the gastrointestinal tract could be an option that might solve these critical issues faced with phytomedicine with potential gastrointestinal benefits such as antidiarrheal, and gastric carcinoma and peptic ulcers properties (De Anda-Flores *et al.,* 2021; Lima *et al.,* 2018). Shatri *et al* (2022) reported the development of Chitosan-Modified PLGA formulations as carriers for antibacterial *G. tenax, L. camara, T. sericea,* and *C. tridens* extracts that have shown

promising physiochemical properties in terms of size, morphology, zeta potential, polydispersity index, and improved release properties. It has been reported that the interactions between polymeric nanoparticles and cells depend on the cell type, as well as on physiochemical properties such as size, zeta potential, and surface charge of nanoparticles which should be constantly monitored during the storage of the formulated nanoparticles (Virlan *et al.,* 2017). Hence, better preservation techniques are required for the prolonged storage of polymeric nanoparticles.

Lyophilization in this context refers to the process of preserving nanoparticles in a powder form by freezing them quickly and then subjecting them to a vacuum which removes ice and water (Izak-Nau *et al.,* 2015). Lyophilization is reported to improve the stability of many acid-labile pharmaceutical products such as antibiotics and phytomedicine that are taken orally (Manohar and Ramesh, 2019). Lyophilization also allows the preservation of nanoparticles in a solid form which enhanced the long-term storage of nanoparticles and helps to prevent cost-intensive cold-chain supply for pharmaceutical products (Ojha *et al.,* 2021). While this could be a better method for the long-term storage of nanoparticles, lyophilization comes with stresses on the formulated nanoparticles as summarised in Figure 1. Nanoparticles are likely to change their physiochemical properties while stored under different conditions and may also 'age' or evolve with time even under optimal storage conditions. Such temporal changes in nanoparticles' properties may affect their toxicity, by increasing or decreasing their dissolution rate, or altering their agglomeration state which can affect bioavailability and in return, this may affect the delivered dose and the efficacy of nanoparticles (Izak-Nau *et al.,* 2015). Hence, using cryoprotectants such as poly (vinyl alcohol) and trehalose that acts as a surfactant when formulating

nanoparticles reduces interaction with the ice surface, and ensures the process and storage stability of the nanoparticles after lyophilization (Zhao *et al.*, 2021). This study aimed at monitoring the physiochemical and biological properties of CMPLGA nanoparticles loaded with extracts of *G. tenax*, *L. camara*, *T. sericea*, and *C. tridens* stored under different conditions after lyophilization.

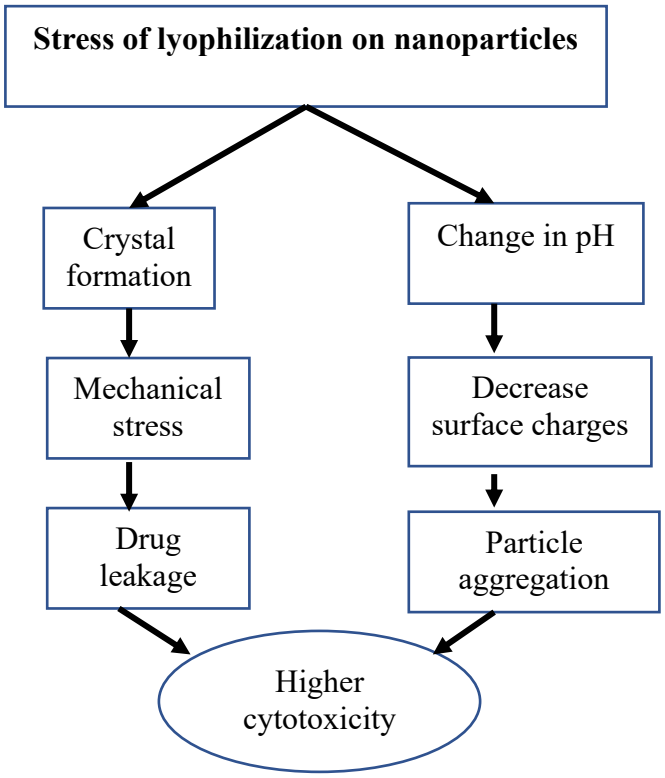

Figure 1: Destabilizing factors and consequences on colloidal NP stability during freezing

6.3 Materials and methods

6.3.1 Preparation of plant-based CMPLGA nanoparticles

Nanoparticles containing antibacterial phytomedicine from *G. tenax, T. sericea, C. tridens,* and *Lantana camara* were formulated in this study. Empty nanoparticles (control) were also formulated in the same manner but without a plant extract, plant-based CMPLGA were prepared using a modified double ($W_1/O/W_2$) emulsion and solvent evaporation method (Arafa *et al.,* 2020; Shatri *et al.,* 2022). Briefly, 120 mg/ml plant extract in methanol was vortexed for 3 minutes. Under stirring 2 % Poly (vinyl alcohol) (PVA) was added to the mixture to form the internal water phase W_1. Exactly 12.5 mg/ml poly (lactic-co-glycolic acid (PLGA) in Dichloromethane (DCM) under stirring was used to prepare the oil phase (O). The internal water phase into the oil content forms the W_1/O primary emulsion. The primary emulsion was homogenized using a Beckman Coulter Optima XE-90 - IVD Ultracentrifuge at 78400 g (relative centrifugal field) for 5 minutes on ice. A W_2 phase was formed using 2.5 ml of 0.1% chitosan 0.1% to 10 ml of 2% PVA. The primary emulsion was added to the external water phase and homogenized at 10 000 rpm for 5 minutes on ice to form a ($W_1/O/W_2$) double emulsion. The double emulsion was left on the stir plate for 24 hours to allow DCM and methanol to evaporate.

6.3.2 Lyophilization of Chitosan-modified PLGA Nanoparticles

The formulated plant-based CMPLGA nanoparticles were *Grewia tenax*-based CMPLGA (CMPLGA(G)), *Terminalia sericea*-based CMPLGA (CMPLGA(T)), *Corchorus tridens*-based CMPLGA (CMPLGA(C)), *Lantana camara* seeds-based CMPLGA (CMPLGA(LS)), *Lantana camara* twigs based CMPLGA (CMPLGA(LT)) and Empty CMPLGA nanoparticles (CMPLGA(EE)). The emulsions prepared were centrifuged for

10 minutes and freeze-dried to form a dry powder. The freezing step involved cooling the sample from room temperature to −50°C using liquid nitrogen, which was followed by primary drying using a freeze drier. In this step, nano-emulsions were dried by increasing the temperature from −20 to 20°C for 72 hours (Chauhan *et al.*, 2016).

6.3.3 Characterization of Chitosan-modified PLGA nanoparticles after lyophilization

Nanoparticle size and zeta potential were assessed by photon correlation spectroscopy using a Malvern zeta sizer/scattering particle size analyzer (Malvern ZS Series Co., UK). To do this, a sample of 2.5 mL was vortexed, placed into an analyzer chamber, and measured. This was done to determine if nanoparticle characteristics were affected by the lyophilization process. This experiment was done at 25 °C in triplicate. The experiment was conducted within 30 days of formulation on nanoparticles stored at 4°C (Azizi *et al.*, 2010).

6.3.4 pH measurement of the lyophilized CMPLGA nanoparticles over six months

The pH of the formulated nanoparticles of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE) was monitored over 6 months for nanoparticles stored at 4 and 25 °C. All the nanoparticles were kept in tightly closed falcon tubes wrapped in parafilm. Briefly, 2 mg of lyophilized nanoparticles were suspended in deionized water and vortexed for 10 minutes at 3000 rpm. The pH of the suspension was measured using a pH meter. The experiment was done in triplicates.

6.3.5 Antibacterial activity of lyophilized CMPLGA nanoparticles

6.3.5.1 Nanoparticle solution preparation

The antibacterial activity of the lyophilized nanoparticles was determined to simulate the behavior of intact nanoparticles over a 24-week storage period at 4 and 25°C. This was done to detect leaking in poor water-soluble nanoparticles such as Chitosan-PLGA nanoparticles which is an indicator for nanotoxicology (Najahi-Missaoui *et al.,* 2020). To prepare the intact nano-solutions (INS) of plant-based CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE), 400 µg nanoparticles were dispensed in 2 ml of deionized water to create a nanosuspension. The nanosuspension was vortexed for 100 minutes and stored for 24 hours at 4°C before the experiment. The antibacterial activity of nanoparticle dissolution (ND) was tested in a simulated intestinal solution using a modified method by Yongsirasawad and Yasurin (2017). This was achieved by dissolving 400 µg of plant-based CMPLGA nanoparticles into 20 µl of DCM. To the mixture with 1ml of 1 mol, $NaHCO_3$ was added and kept for 100 minutes in the fume hood under magnetic stirring to homogenize the simulation and to evaporate out DCM. The pH was maintained at 7.4. The solution was stored for 24 hours at 4°C before the experiment.

6.3.5.2 Bacteria strains preparation

Verotoxigenic Escherichia coli 157:H7 and *Salmonella typhimurium* multi-drug resistant clinical isolates were obtained from children with acute diarrhea at Katutura state hospital (Windhoek, Namibia) (Iikasha *et al.,* 2020). All the bacteria strains were cultured in Mueller Hinton broth (MHB) (Merck, Germany) at 37°C for 24 hours.

6.3.5.3 *In-vitro* antibacterial activity and Minimum Inhibitory Concentration of the INS and ND

The antibacterial activity of CMPLGA INS and ND was determined against *Salmonella spp* and Verotoxigenic *Escherichia coli* 157:H7 multidrug-resistant clinical isolates, using Kirby–Bauer Disk Diffusion Susceptibility test method (Loo *et al.,* 2018) with minor modifications. The antibacterial activity of INS and ND that were stored at 4 and 25 °C was determined within the first 30 days after preparation and after 24 weeks of storage at 4 and 25 °C respectively. This was done to observe the effect of storage temperature on the stability and antibacterial activity of the formulations and to detect leakage. The bacteria strains were spread on the Mueller-Hinton agar (MHA) (Merck, Germany) using a sterile cotton swab. A sterile blank antimicrobial susceptibility disk was loaded with 10 µl of the 200 µg/ml respective nanoparticles solutions. The disc was air-dried, placed on the agar plate, and incubated at 37°C for 24 hours. The zone of inhibition was observed after 24 hours of incubation. Tetracycline has used as positive control while empty nanoparticles (CMPLGA (E)) were used as negative controls. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition around the disc loaded with antibiotics and classified as strong (zone ≥13 mm), moderate (zone ≥ 7 mm), and inactive (zone ≤ 6 mm) (Iikasha *et al.,* 2020; Dulger *et al.,* 2004), The MIC and MBA tests were only determined for nano dissolution (ND). MIC test was performed on a 96-well round-bottom microtiter plate using a standard resazurin-based 96-well plate microdilution method while the MBC test was performed on the Muller Hinton agar plates (Shatri and Mumbengegwi, 2022; Loo *et al.,* 2018). The bacterial inoculums were adjusted to the concentration of 10^6 CFU/ml. For the MIC test, 100 µl of the ND at a concentration of 200 µg/ml was added and diluted twofold with 100 µl of Muller Hinton broth starting from column 3 to column 12. Column 3 of the microtiter plates contained the highest

concentration of ND, while column 12 contained the lowest concentration. Column 1 served as the negative control (only medium) and column 2 served as the positive control (medium and bacterial inoculums). The experiments were conducted in triplicate. Briefly, 30 µl of resazurin and 10 µl of the bacteria inoculum were added to each well of the microtiter plate and incubated at 37°C for 24 hours. Any color changes were observed. Blue/purple color indicated no bacterial growth while pink/colorless indicated bacterial growth. The MIC value was taken at the lowest concentration of antibacterial agents that inhibits the growth of bacteria (color remained blue). The MBC was defined as the lowest concentration of the antibacterial agents that completely kill the bacteria. MBC test was performed by plating the suspension from each well of microtiter plates onto the Muller Hinton agar plates. The plates were incubated at 37°C for 24 hours. The lowest concentration with no visible growths on the Muller Hinton agar plates was taken as the MBC value.

6.3.6 Cytotoxicity assay

6.3.6.1 Cell lines

The fibroblast NIH-3T3 cells were grown at 37 °C in humidified 5%-CO_2 and 95%-air atmosphere in Dulbecco's modified eagle medium supplemented with 10% (vol/vol) inactivated bovine serum, penicillin G, and streptomycin (100 mg/l). Before a uniform (80-90 %) confluence monolayer of cells was formed, cells were freed from the surface of the culture flask by a 0.25% solution of trypsin and were sub-cultivated two times a week until the 8^{th} passage. The NIH-3T3 cells were cultivated flask at a density of 6×10^4 / ml medium and incubated for 48 hours before the experiments.

The cell density was calculated using the equation below:

Total cells/ml= Total cells counted x (Dilution factor/ Number of squares) x 1×10^4

6.3.6.2 MTT Assay

The cytotoxic effect of the prepared-on cells was detected in vitro using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (Sigma, Chem, St. Louis, MO), following the procedure as described by Wang et al. (2019) with modifications. Cell viability was evaluated using thiazolyl blue tetrazolium bromide (MTT), which indicates the metabolic activity of cells. The experiment was performed in 96-well microplates. The cells were seeded at a density of $2.85x10^5$ cells per well. The Nanoparticle suspensions of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE) were dissolved in DMSO (stock solution 0.5%), and subsequently diluted in medium to the final concentration of 100 μg/ml - 3.125 μg/ml and after 24 hours they were added to the cells. Microplates were cultivated for 48 hours in a thermostat at 37 °C and a 5% CO_2 atmosphere. After incubation 30 μl of 5000 μg/mL thiazolyl blue tetrazolium bromide in phosphate-buffered saline, pH=7.4 was pipetted to each well and left to incubate for further 2 hours in the dark. Following this, the medium with MTT solution was removed. Formazan crystals in viable cells were dissolved by adding Dimethyl sulfoxide (DMSO) to each well. Microplates were shaken for 15 minutes. The plates were placed in the spectrophotometer and shaken for 5 minutes. The optical density was determined at 570 nm using a multi-well spectrophotometer. The percentage of cell viability was calculated as per the following formula:

Cell viability (%) = ((Absorbance of sample- Absorbance of blank)/ (Absorbance of control- Absorbance of Blank)) × 100

The 50% Inhibitory concentration (IC_{50}) was defined as the nanoparticle concentration required for the reduction of cell viability by half. The IC_{50} value and the standard error mean (SEM) were calculated using a non-linear regression curve contained in Excel (Concentrations 100- 3.125 μg/ml). The threshold of cytotoxicity of the nanoparticles was set at the IC50 of 50 μg/mL.

6.4 Statistical analysis

The data were analyzed using GraphPad Prism (version 7, 2000, USA). Means were calculated for all variables in the study and the least square means were used to determine significant differences. Findings with a *p*-value of < 0.05 were statically significant. One-way ANOVA was used to determine the statistical difference in antibacterial activity between INS and ND nanoparticles stored under different temperatures over 24 weeks.

6.5 Results and discussion

6.5.1 The effect of lyophilization on plant-based CMPLGA nanoparticle size, zeta potential, and polydispersity index

6.5.1.1 The effect of lyophilization on nanoparticle size

Lyophilization is one of the methods frequently used when preparing therapeutic dry powders, it is reported to improve the formulation stability, however, in some cases, it might affect the quality of the therapeutic product (Mohammady *et al.*, 2020). Table 1 shows the size of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE) plant-based nanoparticles before and after lyophilization. In this study, the nanoparticle sizes of all the formulations were significantly increased after lyophilization (Table 1). However, despite the increase in size after lyophilization, the nanoparticles formulated remained within the nano-size range,

with the smallest being 1728±76.07nm for CMPLGA(L) and the largest being 4301±222.8 nm for CMPLGA(LT). Table 1 shows the changes in size between the empty nanoparticle (EE) and the formulations of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), and CMPLGA(LT) (Table 1) after lyophilization. The increase in nanoparticle size is often linked to aggregation which is reported in other studies that formulated PLGA nanoparticles (Degobert, and Aydin, 2021). Although there is a reported increase in nanoparticle sizes in this study, other studies have reported that the use of cryoprotectants such as trehalose and PVA before lyophilization could offer sufficient protective properties despite the nanoparticle's increase in size (Almalik *et al.,* 2017)

Table 1: A comparison in particle size of selected formulations before and after lyophilization of plant-based CMPLGA nanoparticles

Nanoparticles	**Pellet suspension (nm) before lyophilization**	**Pellet suspension (nm) after lyophilization**
CMPLGA(G)	528.6±502.5	3301±165.2
CMPLGA(T)	1514±864.2	2362±12.98
CMPLGA(C)	1505±301.8	3162±52.50
CMPLGA(LS)	414±202.8	1728±76.07
CMPLGA(LT)	634.4±472.8	4301±222.8
CMPLGA(EE)	574.6±151.6	2807±29.64

CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3.

6.5.1.2 The effect of lyophilization on nanoparticles' zeta potential

In this study, the net surface charge of the CMPLGA nanoparticles had a slight decrease in all formulations after lyophilization except with CMPLGA(LT) which rather showed a

slight increase from 4.89±3.24 to 6.83±3.51 mV after lyophilization. (Table 2). However, all the changes in the zeta potential of the plant-based CMPLGA nanoparticles were statistically insignificant ($p>0.05$). The empty CMPLGA (CMPLGA(EE)) nanoparticles used as a control showed a statistically significant change in the zeta potential of 36.6±3.32 to 6.36±4.74 mV, $p> 0.05$ after lyophilization (Table 2). Since the zeta potential values of the plant-based CMPLGA nanoparticles remained in a close range even after lyophilization that is a sign of electrical stability and stability of surface morphology. This indicates the suitability of the lyophilization process for the storage of these nanoparticles. While the empty nanoparticles (CMPLGA(EE)) with a significant change in zeta potentials tend to coagulate leading to poor physical stability (Varenne *et al.,* 2015).

Table 2: A comparison of the zeta potential of nanoparticles before and after lyophilization of plant-based CMPLGA nanoparticles

Nanoparticles	**Pellet suspension before lyophilization (mV)**	**Pellet suspension after lyophilization (mV)**
CMPLGA(G)	13.2±3.89	5.38±4.80#
CMPLGA(T)	1.66±3.31	-13.2±5.48#
CMPLGA(C)	-2.05±3.64	-3.11±4.79#
CMPLGA(LS)	12.1±4.87	6.87±3.51#
CMPLGA(LT)	4.89±3.24	6.83±3.51#
CMPLGA(EE)	36.6±3.32	6.36±4.74*

CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, #: $p<0.05$ after lyophilization; *: $p> 0.05$ after lyophilization; n=3.

6.5.1.3 The effect of lyophilization on CMPLGA nanoparticles' polydispersity index

In this study, the polydispersity index (Pdi) of all the nanoparticles ranged from 0.2 to 1 and the Pdi change for CMPLGA (LT, G, and LS) formulations was ≤ 0.7, which was statistically insignificant ($p < 0.05$). Studies have shown that when the Pdi value is less than 0.7, this indicates greater stability for a nano-delivery/colloidal system since the Pdi values influence the drug release kinetics and cellular uptake in drug-delivery applications of nanoparticles (FDA, 2018; Pdi (Danaei *et al.*, 2018). The Pdi of nanoparticles is also a highly important physical characteristic to be considered when creating pharmaceutical-grade products since it shows the bulk properties, product performance, processability, stability, and appearance of the end product (Danaei *et al.*, 2018).

Table 3: A comparison in Pdi of nanoparticles before and after lyophilization of plant-based CMPLGA nanoparticles

Nanoparticles	**Pellet suspension before lyophilization**	**Pellet suspension after lyophilization**
CMPLGA(G)	0.353±0.00	0.747±0.00*
CMPLGA(T)	0.503±0.00	1.000±0.00
CMPLGA(C)	0.203±0.00	1.000±0.00
CMPLGA(LS)	0.192±0.00	0.622±0.00*
CMPLGA(LT)	0.281±0.00	0.231±0.00*
CMPLGA(EE)	0.439±0.00	1.000±0.00

CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3,

*: $p < 0.05$ after lyophilization

6.5.2 The effect of storage temperature on the pH of the plant-based CMPLGA nanoparticles

In this study, the pH of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE) nanoparticles stored at 4 and 25 °C was monitored

over 12 months at 2 months' intervals (Figures 2 A and B respectively). Although the pH of all CMPLGA nanoparticles decreases with time. The initial pH from 5 to 4 in CMPLGA nanoparticles stored at 4°C was statistically insignificant after 12 months of storage ($p< 0.05$). However, the CMPLGA nanoparticles stored at 25°C showed a significant decrease in pH from 5 to 2 in most nanoparticles after 12 months of storage ($p> 0.05$) (Figure 2 B). It has been reported that a decrease in entrapment efficiency which could be an indicator of PLGA degradation commonly leads to a drop in pH values (Stecanella *et al.,* 2013; Rapier *et al.,* 2021), which was observed in the formulations stored at 25°C. Hence, the suitable storage temperature of the plant-based CMPLGA nanoparticles is 4 °C since storage temperature causes a significant decrease in the storage temperature of the plant-based PLGA nanoparticle. It can also be seen based on the change in pH over time that the longer the storage period for nanoparticles, the more acidic the nanoparticles become. This could be because a minimal quantity of plant extracts was released during storage due to polymer degradation in the aqueous environment (Aveamescu *et al.,* 2017).

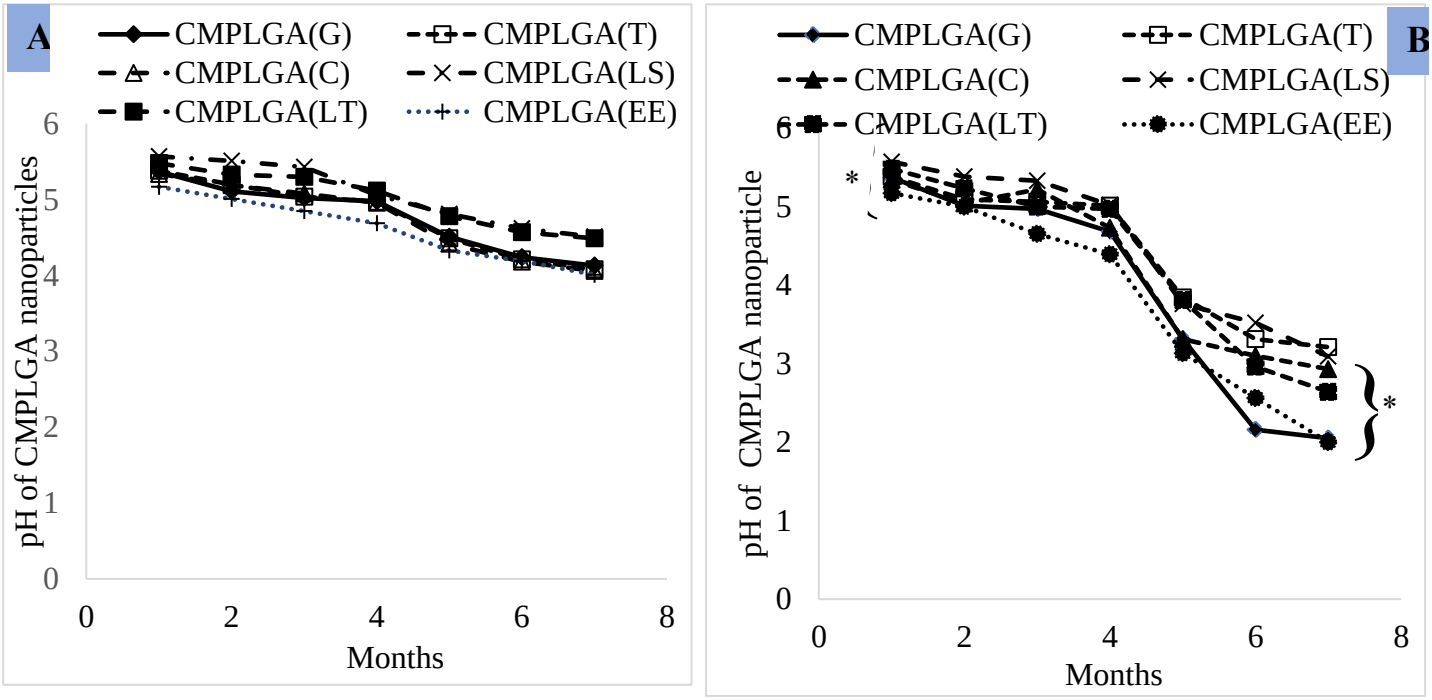

Figure 2: A: The pH measurement of the lyophilization nanoparticles over 6 months stored at 4°C and B: the pH measurement of the lyophilization nanoparticles over 6 months stored at 25 °C CMPLGA: plant-based Chitosan-Modified PLGA nanoparticles; n=3

6.5.3 Comparing the antibacterial activity of the lyophilized plant-based CMPLGA INS and ND nanoparticles stored at different temperatures over 24 weeks

6.5.3. 1 Antibacterial activity of ND for CMPLGA nanoparticles stored at 4 °C and 25°C after 4 and 24 weeks against *Salmonella*

Monitoring the antibacterial activity of ND after drug encapsulation is important as this will provide useful information regarding the efficacy and payload of the final formulated nanoparticles at the targeted release point (Lu *et al.*, 2021). Moreover, monitoring the effect of storage temperature and the storage period will also be a good indicator for leakage of nanoparticles by monitoring the antibacterial activity of intact nano-solution

(INS) over a while and provides useful information regarding the safe place of storing the nanoparticles as well as the shelf life of the nanoparticles.

Comparing the antibacterial activity of nanoparticle dissolutions stored at 4 and 25°C for 4 and 24 weeks, overall, the CMPLGA nanoparticles stored at 4°C showed higher antibacterial activity than those stored at 25°C. The nanoparticles stored at 4°C and 25 °C respectively for 4 weeks both showed antibacterial activity with CMPLGA(LS) and CMPLGA(C) showing potent antibacterial activity with average inhibition zones of 19.67±0.58 mm and 18.33±0.58 mm against multidrug-resistant clinical *Salmonella.* This was similar to the positive control tetracycline which showed an average inhibition of 18.0±1.00 mm (Figure 4 A-B). The antibacterial activity observed in nanoparticles stored at 4°C and 25 °C respectively for 4 weeks against multidrug-resistant Verotoxigenic *Escherichia coli* 157:H7 also showed potent antibacterial activity with average inhibition zones of 15.67±0.58 mm and 14.33±0.58 mm (Figure 5 A-B). However, while there was a significant decrease in the antibacterial activity of nanoparticles after 24 weeks of storage at 25 °C, there was no significant difference in the antibacterial activity of nanoparticles stored at 4 °C after 24 weeks (Figure 4 C-D and Figure 5 C-D) against both *Salmonella* and Verotoxigenic *Escherichia coli*. DCM and CMPLGA (EE) that were used as negative controls did not show any antibacterial activity (Figure 3). This shows that the antibacterial activity observed is not from the components used in the formulation such as solvents and polymer but from the plant extract encapsulated. Statistically, CMPLGA(T), CMPLGA(G), and CMPLGA(LS) showed a significant difference in antibacterial activity of nanoparticles stored at 4 and 25°C for 4 weeks in comparison to positive control tetracycline with a $p < 0.001$ and CMPLGA(LS) $p< 0.05$. However, there

was no significant difference in antibacterial activity when compared to positive control tetracycline $p > 0.05$ after 4 weeks of storage at 4 and 25°C. Studies have shown that the storage time and temperature may influence the concentration of the chemical components present in medicinal plants and poor storage conditions may result in active compounds deteriorating and this reduces the effectiveness of medicinal plants (Cristiane *et al.*, 2018).

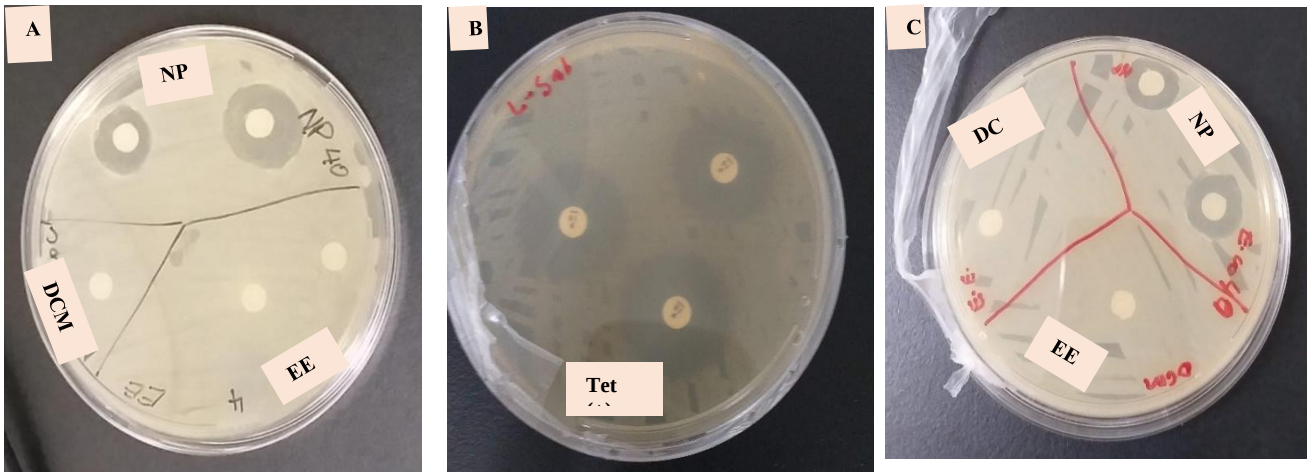

Figure 3: Antibacterial effect of A) NP: CMPLGA (LS), DMC: Dichloromethane, EE: CMPLGA(EE) at 4°C; B) Tet (+): tetracycline; C) NP: CMPLGA (C), DMC: Dichloromethane, EE: CMPLGA(EE) at 25°C on clinical *Salmonella* isolate

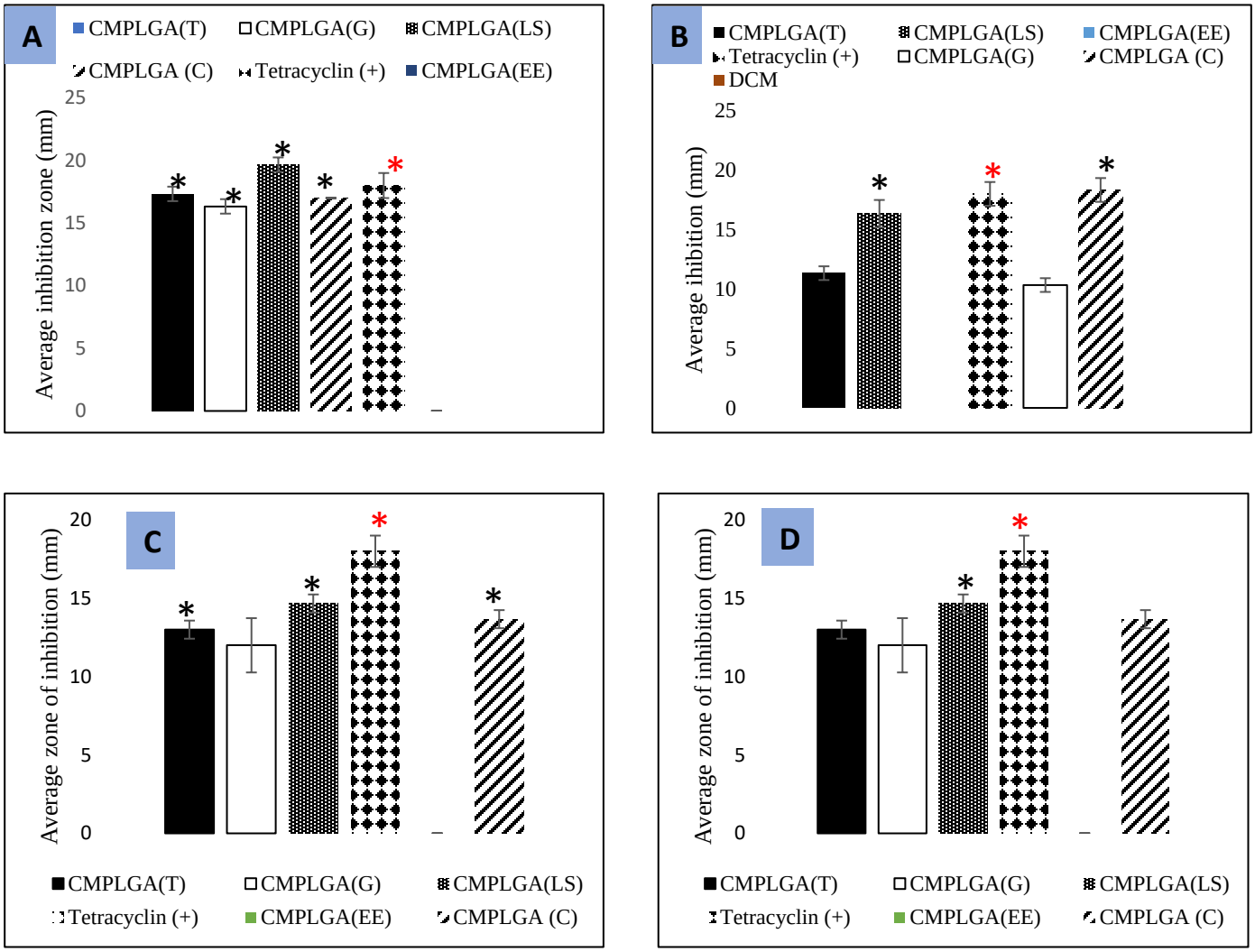

Figure 4: Antibacterial activity of ND of nanoparticles stored at A: 4 °C for 4 weeks, B: 25 °C for 24 weeks, C: 4°C for 24 weeks, and D: 25°C for 4 weeks against multidrug-resistant clinical *Salmonella* isolate. CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3, *p>0.005 when compared to tetracycline; p>0.005 for comparison between A, B, and C and D

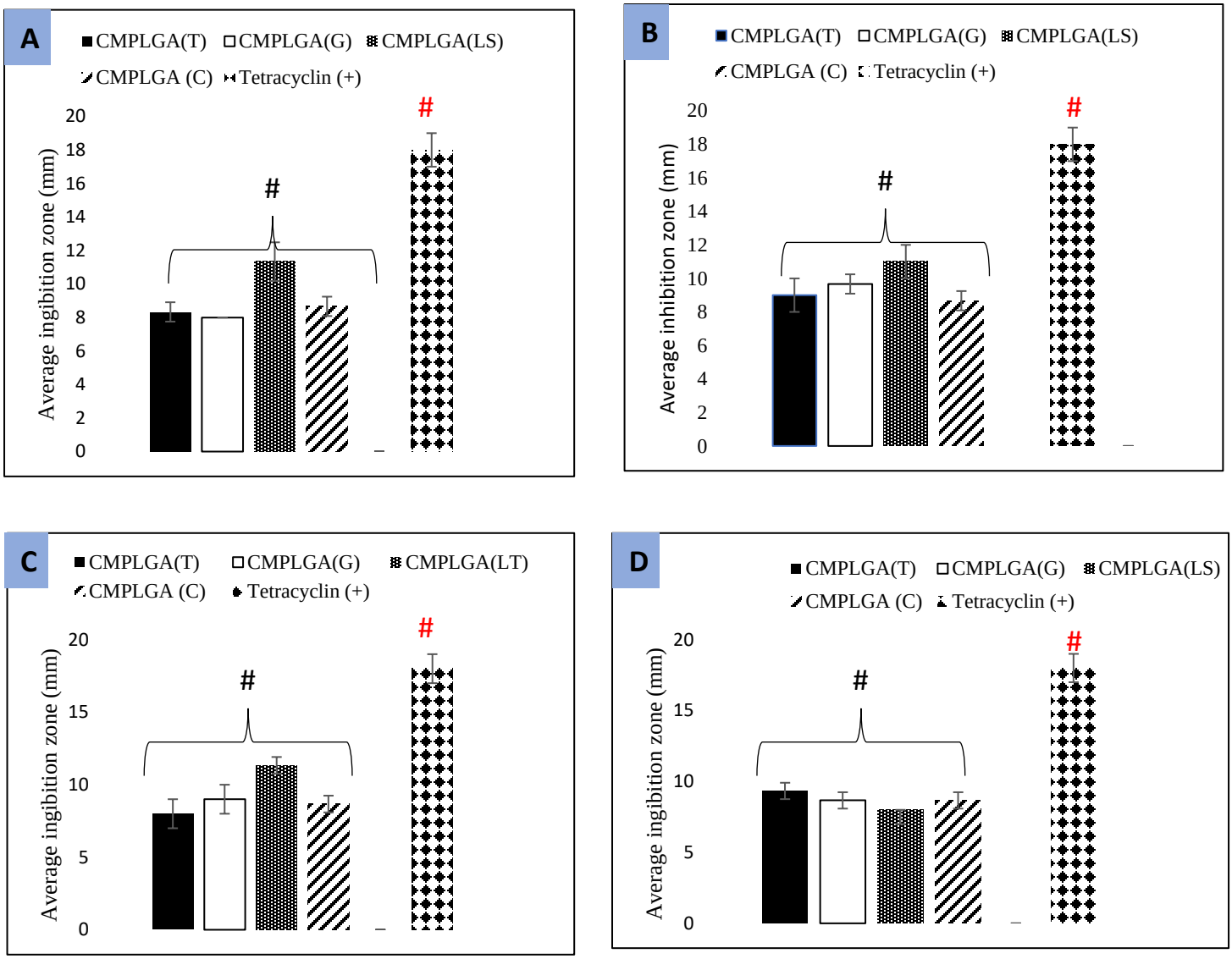

Figure 5: Antibacterial activity of ND of nanoparticles stored at A: 4 °C for 4 weeks, B: 25 °C for 4 weeks, C: 4°C for 24 weeks, and D: 25°C for 24 weeks against multidrug-resistant clinical Verotoxigenic *Escherichia coli* 157:H7 isolate. CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3, #: CMPLGA nanoparticles' $p>0.005$ when compared to tetracycline; $p< 0.005$ for comparison between A, B, C, and D.

6.5.3. 2 Antibacterial activities of plant-based CMPLGA INS nanoparticles stored at 4 °C and 25°C for 4 and 24 weeks against *Salmonella*

Comparing the antibacterial activity of INS nanoparticles stored over 24 weeks was done to detect leaking by monitoring an increase in antibacterial activity over time. For INS nanoparticles stored at 4 and 25°C for 4 weeks, overall, the CMPLGA nanoparticles stored at 4°C and 25°C showed similarity in antibacterial activity. The intact nano-solutions stored at 4°C and 25 °C respectively for 4 weeks both showed antibacterial activity with CMPLGA(LS) and CMPLGA(C) showing moderate antibacterial activity with average inhibition zones of 11.33±0.58 and 11.00±0.58 mm against multidrug-resistant clinical *Salmonella.* This was however significantly lower than the antibacterial activity of the positive control tetracycline which showed an average inhibition of 18.0±1.00 mm $p < 0.05$ (Figure 6 A-B). The antibacterial activity observed in nanoparticles stored at 4°C and 25 °C respectively for 4 weeks against multidrug-resistant Verotoxigenic *Escherichia coli* 157:H7 showed moderate antibacterial activity with average inhibition zones of 11.33±0.58 mm and 14.33±0.58 mm (Figure 7 A-B) which were significantly different from the positive control tetracycline 20.3±0.58 mm, $p< 0.001$. However, there was no statistically significant increase in the antibacterial activity of nanoparticles after 24 weeks of storage at 25 °C, and nanoparticles stored at 4 °C after 24 weeks (Figure 6 and 5 C-D) against both *Salmonella* and Verotoxigenic *Escherichia coli*. This could indicate that there was no significant leakage even in nanoparticles stored at room temperature $p >0.05$.

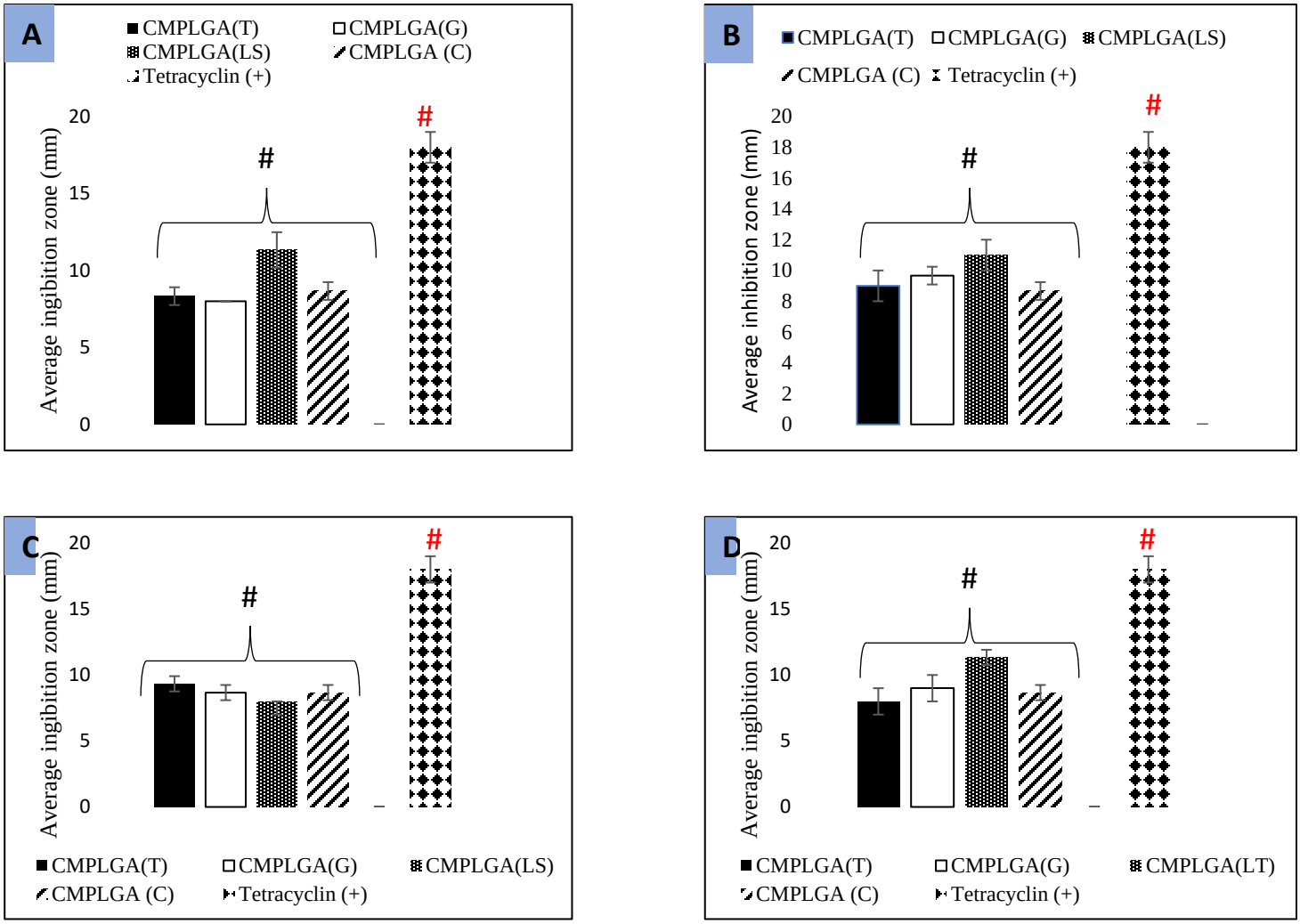

Figure 6: Antibacterial activity of INS of nanoparticles stored at A: 4 °C for 4 weeks, B: 25 °C for 4 weeks, C: 4°C for 24 weeks, and D: 25°C for 24 weeks against multidrug-resistant clinical *Salmonella* isolate. CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3, p>0.005 except for CMPLGA(LS) compared to tetracycline.

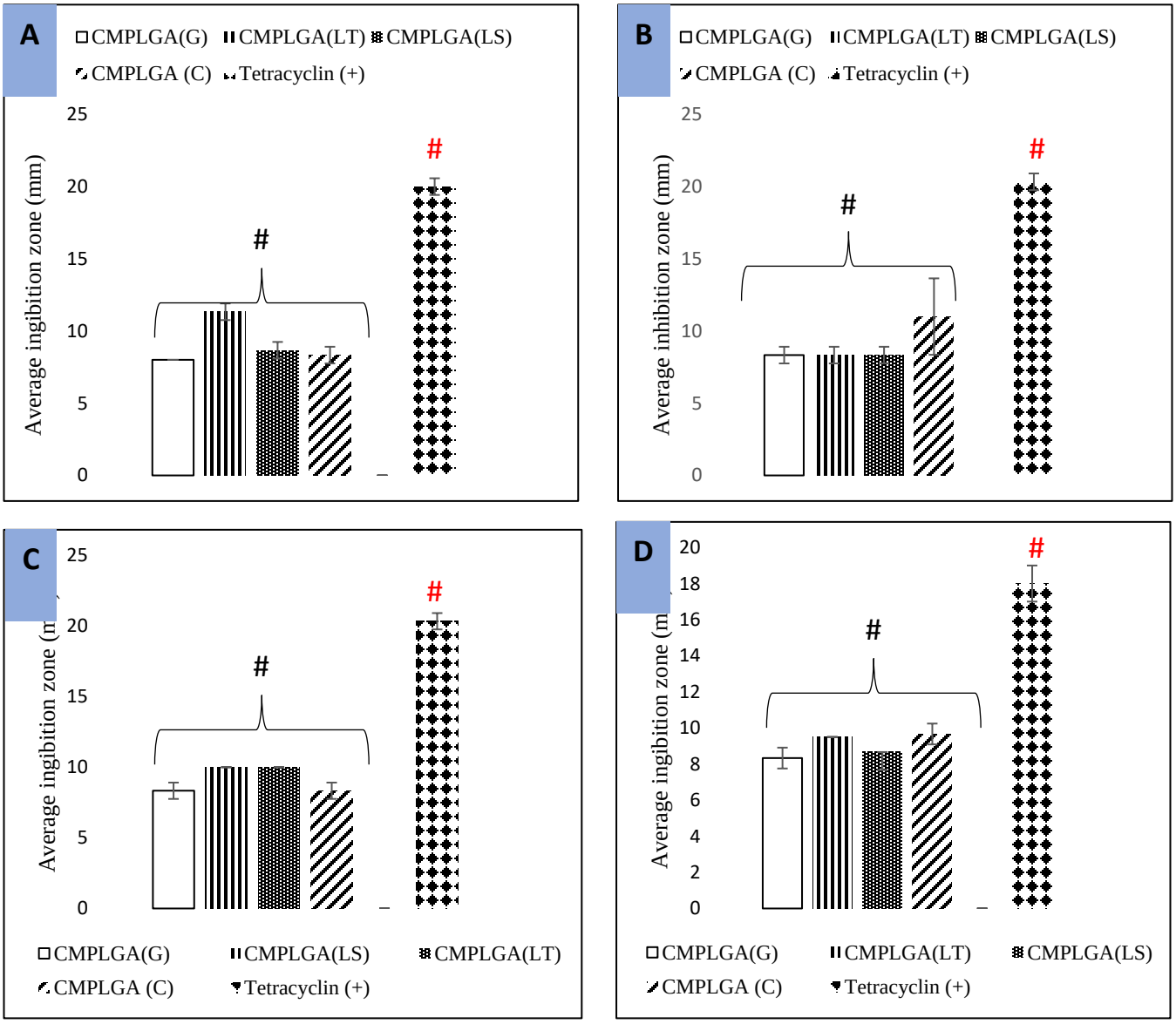

Figure 7: Antibacterial activity of INS of nanoparticles stored at A: 4 °C for 4 weeks, B: 25 °C for 4 weeks, C: 4°C for 24 weeks, and D: 25°C for 24 weeks against multidrug-resistant clinical Verotoxigenic *Escherichia coli* 157:H7isolate. CMPLGA: Chitosan-Modified PLGA, Data presented as Mean ± Standard deviation, n=3, p>0.005 except for CMPLGA(LS) compared to tetracycline.

Overall, based on the antibacterial analysis of INS and ND stored at 4 and 25°C, in all CMPLGA nanoparticles, the nanoparticles stored at 4°C showed potent antibacterial activity compared to those stored at 25°C in nano-dissolutions (ND), however, there was no significant difference in antibacterial activity of intact nano-solution indicating minimal to no leakage. There was also a significant difference in the antibacterial activity of different nanoparticles to *Salmonella* and *E. coli* ($p < 0.001$). When evaluating the potency of the ND verse a clinically used antibiotic, the nanoparticles were as potent as the positive control against *Salmonella*. The antibacterial activity of the ND of the plant-based CMPLGA nanoparticles against clinical *Salmonella* and Verotoxigenic *Escherichia coli* 157:H7 decreased with a prolonged storage period, especially for nanoparticles stored at 25°C. However, for INS nanoparticles stored at 25°C, there was no statistically significant increase in antibacterial activity of the INS with time which could be an indication of no leakage. This could mean that while the temperature of 4 °C is more suitable for the storage of plant-based CMPLGA nanoparticles, the temperature of 25°C can also be used for the short-term storage of plant-based CMPLGA nanoparticles.

The potent antibacterial activity observed in the ND tested at 7.4 pH is an important property especially because it shows potent antibacterial activity (payload) of the released antibacterial plant extracts within the ileum where *Salmonella* and Verotoxigenic *Escherichia coli* 157:H7 resides. It is also important to note that, by looking at the averages of the inhibition zone, encapsulating the extract in polymers has enhanced the antibacterial activity of the extracts against clinical *Salmonella* and Verotoxigenic *Escherichia coli* 157:H7. This is consistent with the findings of Lu et al. (2021) and Anand

et al. (2019) who reported that the use of nanocarriers enhanced the antibacterial effect of tilmicosin and ciprofloxacin respectively.

The results of the MIC and MBC of CMPLGA nanoparticles and controls are shown in table 4. Among all the ND of plant-based CMPLGA nanoparticles stored at 4 °C and 25 °C, The MPLGA(LS) stored at 4°C showed the lowest MIC and MBC of 6.25 µg/ml and 25.0 µg/ml respectively against *Salmonella* and while CMPLGA (G) showed the lowest MIC and MBC. Moreover, CMPLGA(G) stored at 4°C nanoparticles showed the lowest MIC and MBC of 12.5 µg/ml against Verotoxigenic *Escherichia coli* 157:H7. This indicates that 4 °C is the best storage of the plant-based CMPLGA nanoparticles. As reported in this study, other studies have shown that the use of chitosan in formulating nanoparticles decreases the MIC of an actual antibacterial pharmaceutical compound by 50% (Nguyen *et al.*, 2016). Moreover, the enhanced antibacterial activity of CMPLGA nanoparticles against gram-negative observed in this study may be due to the interactions between the negative charge of the gram-negative Verotoxigenic *Escherichia coli* 157:H7 and *Salmonella* cell membranes and the positive charge of chitosan and the antibacterial properties of the secondary metabolites present in the plant extracts (Anand *et al.*, 2019; Shatri et al., 2022). Negatively charged nanoparticles such as those of CMPLGA(T)/ (C) may show poor affinities with the gram-negative pathogens and this may reduce their effectiveness in eliminating the pathogens. A study by Li, *et al.* (2019) also showed that negatively charged magnetic nanoparticles did not show affinities toward *E. coli*. However, positively charged nanoparticles synthesized in this study have a higher chance of capturing the negatively charged bacteria via electrostatic attractions, which can enhance the effectiveness of the nanoparticles in eliminating pathogens.

Table 4: The MIC and MBC of CMPLGA lysed and un-lysed nanoparticles against clinical *Salmonella* and Verotoxigenic *Escherichia coli* 157:H7 stored at 4 and 25 °C

	***Salmonella* isolates**				**Verotoxigenic *Escherichia coli* 157:H7**			
Nanoparticles	MIC of ND 24 weeks (4 °C) (µg/ml)	MBC of ND 24 weeks (4°C) (µg/ml)	MIC of ND 24 weeks (25°C) (µg/ml)	MBC of ND 4 24 weeks (25°C) (µg/ml)	Average MIC of ND 24 weeks (4°C) (µg/ml)	MBC of ND 24 weeks (4°C) (µg/ml)	Average MIC of ND 24 weeks (25°C) (µg/ml)	MBC of ND 24 weeks(25°C) (µg/ml)
CMPLGA(G)	50.0±0.00*	100±0.00	100±0.00*	100±0.00	62.5±0.00	62.5.0±0.00	100.0±0.00	100.0±0.00
CMPLGA(T)	12.5±0.00*	25.0±0.00	50.0±0.00*	50.0±0.00	-	-	-	-
CMPLGA (C)	50.0±0.00	50.0±0.00	50.0±0.00	50.0±0.00	50.5±0.00*	100±0.00	200.0±0.00*	200.0±0.00
CMPLGA(LS)	6.25±0.00*	6.25±0.00	25.0±0.00*	25.0±0.00	12.5±0.00*	12.5±0.00	100.0±0.00*	200.0±0.00
CMPLGA(LT)	-	-	-	-	25.5±0.00*	25.5±0.00	100.0±0.00*	100.0±0.00
Tetracycline (+)	0.23±0.00	0.23±0.00	0.23±0.00	0.23±0.00	0.23±0.00	0.23±0.00	0.23±0.00	0.23±0.00

CMPLGA: Chitosan modified PLGA; MIC: Minimum Inhibitory concentration; MBC: Minimum Bacteria Concentration; ND: nano-dissolutions. Data presented as Mean± Standard deviation, n=3, *p< 0.05 MIC 4 °C verse 25°C

6.5.4 *In vitro* cytotoxicity studies on NIH-3T3 mouse embryo fibroblast cell line

While nanoparticle encapsulation has the advantages of increased bioavailability and biocompatibility which could increase the drug uptake into cells, this, in turn, could also increase drug cytotoxicity (Herdiana *et al.*, 2021). Although the safety of PLGA has been widely recognized due to its biocompatibility and biodegradability there is limited data on the safety of CMPLGA nanoparticles.

To assess the cytotoxicity of the nanoparticles on the NIH-3T3 mouse embryo fibroblast cell line, cell viability experiments were performed by the MTT assay within 48 hours after incubation. MTT predicts cytotoxicity by evaluating mitochondrial and nonmitochondrial enzymatic activity. The cytotoxic activities of the CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), CMPLGA(LT), and CMPLGA(EE) nanoparticles were determined at various concentrations on 3T3 mouse embryo fibroblast cells. Figure 8 A-E showed the effect of plant extract-loaded CMPLGA nanoparticles and blank CMPLGA nanoparticles on the proliferation of 3T3 mouse embryo fibroblast cells. The values for cell viability with plant-based CMPLCA nanoparticles at the highest concentration of 100μg/mL (Figure 8) were 28.22±0.09% for CMPLGA(G), 34.04±0.07% for CMPLGA(T), 33.15±0.03% for CMPLGA(C), and 33.48±0.08% for CMPLGA(LS). It is important to notice that there was also a toxicity of 67.57±0.07% observed in cells treated with CMPLGA(EE). At the lowest concentration of 1.56 μg/mL for all CMPLGA nanoparticles, the percentage viability was between 87.7±0.08% and 91.50±0.156% which indicates that the nanoparticles have less toxicity and have a greater level of safety *in vitro*, at the lower concentrations. This study demonstrates the in vitro cytotoxicity profile of CMPLGA(G), CMPLGA(T), CMPLGA(C), CMPLGA(LS), and CMPLGA(LT) using 3T3 a mouse embryo fibroblast cell line. The results showed that all CMPLGA nanoparticles show concentration-dependent cytotoxic effects against the 3T3 cell line.

Moreover, among the four studied plant-based CMPLGA nanoparticles, CMPLGA(C) showed the highest average half-maximal inhibitory concentration (IC_{50}) values of 76.6±0.09 μg/mL after 48 hours (Table 5) which indicates that it has lower cytotoxicity

than the other nanoparticles. Meanwhile, CMPLGA(S) showed a lower IC_{50} of 66. 7±0.05±0.05 µg/mL as depicted in table 5. CMPLGA(EE) alone showed an IC_{50} of 88.1±0.07. Therefore, since the IC_{50} of all the nanoparticles is above 50 µg/mL which was set as the threshold for cytotoxicity of the nanoparticles, the extracts are considered not toxic. It is however important to evaluate the cytotoxicity of the nanoparticles in a gastric or intestinal cell line. Although the NIH-3T3 mouse embryo fibroblast cell line is widely used in cytotoxicity studies due to it can secrete several important growth factors into the medium, which help maintain pluripotency, and they provide a cellular matrix for cells to grow, it is important to evaluate the cytotoxicity of the nanoparticles in other cell lines such as Human intestinal epithelial cell lines (T84, Caco-2, and HCT-8) as they may provide more details on the cytotoxicity of the cell plant based CMPLGA nanoparticles specifically to gastrointestinal cells (Hurley *et al.,* 2016).

While the cytotoxicity of tetracycline used as a positive control for antibacterial test in this study was not determined, a study by Fuoco, 2015 looking at the toxicity of tetracycline and its derivatives on human keratinocytes showed that tetracycline was toxic with IC_{50} of 10 µM under the light. Their MTT test key was set as IC_{50} under 10 µM was considered extremely cytotoxic; the range between 50 and 100 µM is considered moderately cytotoxic. Another study by Sog *et al.* (2014) looked at 4 analogs of tetracycline's toxicity to human myeloid HL-60 cell line, in cells treated with concentrations between 0.5, 1, 2.5, 5, 10, 25, 50, and 100 µg/ml the IC_{50} raged between 1.3-9.9 µg/ml. It is therefore important to evaluate the cytotoxicity of the nanoparticles at the MIC concentrations as shown in table 2. In this study, all the plant-based CMPLGA nanoparticles showed potent MIC at the storage temperature of 4°C. Hence, to compare

the understand the toxicity of the nanoparticles stored at 4 °C after 24 weeks the MIC of nanoparticles stored at 4°C after 24 weeks of storage was used. The results showed that the CMPLGA(T) and CMPLGA(S) nanoparticles will have no toxicity at MIC concentrations as shown in table 6. However, CMPLGA(C) and CMPLGA(G) will be used with minimal toxicity at MIC concentration against *Salmonella*. Moreover, to compare the understand the toxicity of the nanoparticles stored at 4 °C after 24 weeks the MIC of nanoparticles against Verotoxigenic *E. coli* 157:H7 for nanoparticles stored at 4°C after 24 weeks, the results showed that the CMPLGA(S) nanoparticles will have no toxicity at MIC concentrations as shown in table 7. However, CMPLGA(G) will be used with moderate toxicity, while CMPLGA(C) will be used with higher toxicity at MIC the concentration against Verotoxigenic *E. coli* 157:H7.

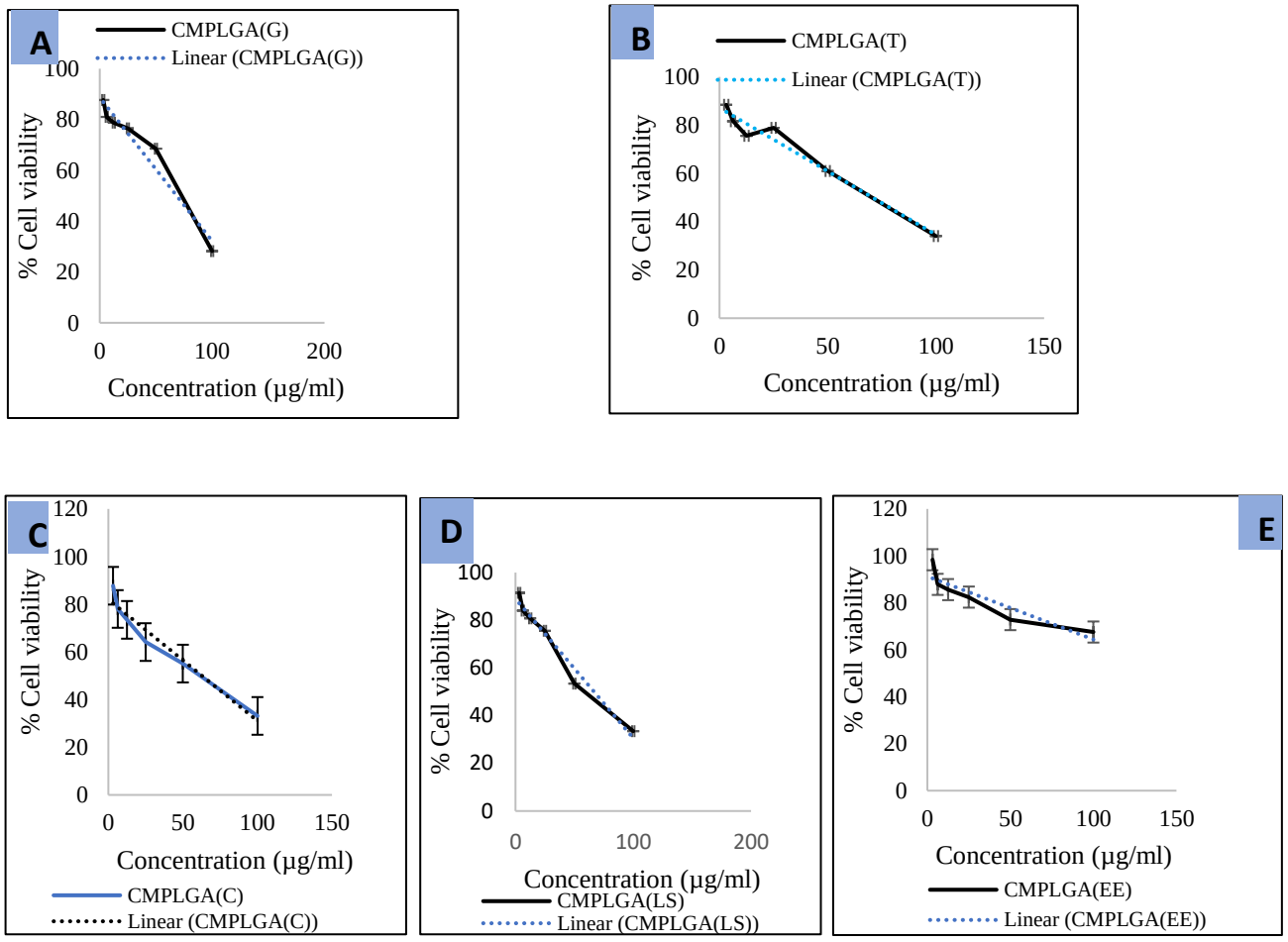

Figure 8: Effect of the A: CMPLGA(G), B: CMPLGA(T), C: CMPLGA(C), D: CMPLGA(LS), nanoparticles stored at 4°C for 24 weeks on NIH-3T3 cell viability after the 48 hours by the MTT (A) The results are expressed as mean and standard error obtained from 4 independent tests with n = 3, $p<0.05$ verses E: CMPLGA(EE)

Table 5: IC_{50} of CMPLGA nanoparticles on 3T3 cell line

CMPLGA Nanoparticle	CMPLGA(G)	CMPLGA(T)	CMPLGA(C)	CMPLGA(S)	CMPLGA(EE)
IC50	68.5±0.05	70.7±0.05	76.6±0.09	66.7±0.05	88.1±0.07

Table 6. Cell toxicity of plant passed CMPLGA nanoparticles at MIC concentrations against *Salmonella*

Treatment	**MIC concentration**	**Percentage (%) cell death at MIC concentration**	**Interpretation**
CMPLGA(G)	50.0	36.5	Minimal toxicity
CMPLGA(C)	50.0	32.6	Minimal toxicity
CMPLGA(S)	6.25	4.7	No toxicity
CMPLGA(T)	12.5	9.4	No toxicity
Tetracycline	0.23	8.8	No toxicity

Key: Key Set for the study: % Cell death at MIC concentration of 0-20 cells= not toxic: 21-45: Minimal toxicity at MIC concentration; 46-60: Moderate toxicity at MIC concentration; 62-100: Higher toxicity at MIC concentration

Table 7. Cell toxicity of plant-passed CMPLGA nanoparticles at MIC concentrations against Verotoxigenic *E. coli* 157:H7

Treatment	**MIC concentration**	**Percentage (%) cell death at MIC concentration**	**Interpretation**

CMPLGA(G)	50.0	45.6	Minimal toxicity
CMPLGA(C)	50.0	65.3	Higher toxicity
CMPLGA(S)	6.25	9.4	No toxicity
CMPLGA(T)	12.5	9.4	No toxicity
Tetracycline	0.23	8.8	No toxicity

Key: Key Set for the study: % Cell death at MIC concentration of 0-20 cells= not toxic: 21-45: Minimal toxicity at MIC concentration; 46-60: Moderate toxicity at MIC concentration; 62-100: Higher toxicity at MIC concentration

6.6 Conclusion

In this study, plant-based CMPLGA nanoparticles were monitored for their physiochemical characteristics and biological activity after lyophilization. The nanoparticles' size increased significantly after lyophilization indicating aggregation. However, the zeta potential and polydispersity index of the plant-based CMPLGA nanoparticles did not significantly change after lyophilization indicating the stability of the nanoparticles. Hence, the exact concentration suitable for preventing aggregation for plant-based PLGA nanoparticles should be determined to see how to decrease the size and Pdi of the nanoparticles after lyophilization. The antibacterial activity of the plant-based CMPLGA nanoparticles was temperature-dependent with the plant-based nano dissolutions showing potent antibacterial activity with the lowest MIC of 6.25µg/ml in nanoparticles stored at 4°C against MDR clinical *Salmonella* and Verotoxigenic *Escherichia coli* 157:H7. However, the nanoparticles were table at 4 and 25 °C since there

was no significant change in antibacterial activity of the intact nanoparticles' solutions over 24 weeks of storage. The antibacterial activity of the nanoparticles was however only tested on single isolates of *Salmonella* and *Verotoxigenic Escherichia coli 157:H7* hence, they should be evaluated against a broader panel of pathogens. Moreover, the plant-based CMPLGA (C) and CMPLGA (S) nanoparticles showed no cytotoxicity in 3T3 mouse embryo fibroblast cells at MIC concentrations while only CMPLGA (S) showed no cytotoxicity at the MIC concentration against *Verotoxigenic E. coli 157:H7,* indicating that these nanoparticles can be used with no cytotoxicity at MIC concentrations.

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CHAPTER 7: OVERALL CONCLUSIONS AND RECOMMENDATIONS

Acute gastroenteritis is responsible for higher morbidity and mortality across all ages worldwide with *Salmonella* and *E. coli* being among the top causes of bacterial gastroenteritis. In Namibia, gastroenteritis is among the top five infectious diseases with a 5% annual mortality rate. Apart from the increase in reports of multidrug resistance to clinically used antidiarrheal treatments, first-line antibiotic treatments also are associated with numerous side effects such as vomiting and severe diarrhea when used in patients with gastroenteritis. Hence there is a need to consider alternative treatments for gastroenteritis. While medicinal plants have been used as alternative and complementary medicine for gastroenteritis globally, there is limited data on the uses of medicinal plants to treat gastroenteritis in Namibia. This limits their potential mainstream use as a complementary and alternative treatment option for gastroenteritis. Moreover, there is no scientific validation or documentation regarding the efficacy and safety of medicinal plants in Namibia. Phytomedicine like currently available antibiotics is faced with delivery barriers within the GI tract, such as degradation of bioactive compounds by the acid pH of the stomach, poor solubility in the gastrointestinal milieu, and poor uptake. As a result, large dosages for decoctions and infusions are required for effectiveness. The aim of the study embodies in this dissertation, was to document the ethnomedicinal uses of plants used for gastroenteritis; to scientifically validate their antibacterial and cytotoxicity properties, to design pH-controlled nanoparticles; and to evaluate the *in vitro* behavior and biophysical properties of the formulated CMPLGA nanoparticles.

Chapter 3 aimed at documenting the ethnobotanical uses and phytochemical composition of plants to treat gastrointestinal conditions in Iikokola village, Omusati region, Namibia. Based on the ethnomedicinal survey conducted with 23 knowledge holders, a total of 19 medicinal plants were documented to treat gastrointestinal conditions and 14 other ailments. All medicines used for treating gastrointestinal conditions were taken orally mainly in form of decoction and infusions. Based on the FVI analysis, *T. sericea, L. camara, G. tenax, A. anthelmintica,* and *C. tridens* were commonly used to treat diarrhea, stomach pain, and vomiting, which are common conditions associated with gastroenteritis. The ethnomedicinal uses of most plants such as *L. camara, S. capense, O. kilimands, P. camestris, C. tridens,* and *G. tenax* to treat gastrointestinal conditions reported in this study are not documented elsewhere in Namibia. Moreover, classes of phytochemical compounds such as alkaloid, steroid, saponin, coumarin, anthraquinone, and flavonoid associated with antidiarrheal properties of plants were detected in macerated extracts of *T. sericea, L. camara, G. tenax, A. anthelmintica,* and *C. tridens*.

Chapter 4 of this study aimed to fill the gap in knowledge regarding the antigram and antibacterial activities of the identified medicinal plants, against clinical and reference strains of *E. coli* and *Salmonella*, as well as the cytotoxicity profiles of the potent antibacterial extracts, to NIH 3T3 cells. Based on the *in vitro* antigram tests conducted on clinical isolates of *Salmonella* and *E. coli* from Namibian children, *Salmonella* and *E. coli* showed 70% resistance while a 60% resistance was observed against reference strains, to clinically used antibiotics. Despite the higher resistance reported in clinical isolates of *Salmonella* and *E. coli*; methanol extracts from *T. sericea, L. camara, G. tenax,* and *C. tridens* showed potent antibacterial activity against the MDR clinical isolates. The lowest

MIC and MBC were reported in *L. camara* organic seed extracts. While the cytotoxicity of the extracts from *T. sericea, L. camara, G. tenax,* and *C. tridens* to mostly cancer cell lines has been reported in other studies, this study is the first to demonstrate the effect of methanol extracts of *T. sericea, L. camara, G. tenax,* and *C. tridens* to NIH 3T3 cells while all extracts showed only minimal cytotoxicity with $IC_{50} \geq 60$ µg/mL, *C. tridens* was less toxic since it showed a higher IC_{50} of 91.50±0.14 µg/mL. This shows that the antibacterial crude extracts are safe for *in vitro* use against NIH 3T3 cells and are likely to also be safe for *in vivo* use.

Chapter 5 aimed to design and characterize the pH-dependent Chitosan modified poly (lactic-co-glycolic acid) (CMPLGA) nanoparticles encapsulating antibacterial plant extracts of *T. sericea, L. camara, G. tenax,* and *C. tridens*. This was done to attempt to observe whether encapsulation improves the in vitro biophysical properties of the novel nanoparticles in gastric and intestinal simulations. The formulated extracts demonstrated high payload uptake since all formulations showed an encapsulation efficacy of ≥ 80%. Moreover, the results show that encapsulating the antibacterial plant extracts in CMPLGA has significantly improved the *in vitro* release properties of the extracts in simulated gastric and intestinal fluids (without enzymes) at pH of 1.2 and 7.4 respectively. There was a decrease of over 87.8% in levels of plant extracts in simulated fluids within two hours when non-encapsulated, this has alluded to instability at low pH. However, for the plant extracts encapsulated in CMPLGA nanoparticles, there was insignificant extract release at pH 1.2 (<2 %), and a significant release of extract at a pH of 7.4 (>50%) over 72 hours. The CMPLGA nanoparticles loaded with *L. camara* phytomedicine showed a significant and sustained release profile at 7.4 pH compared to other nanoparticles. This

means that the polymers could protect the encapsulated antibacterial plant extracts from the gastric pH (acidic).

Chapter 6 aimed to determine the effect of lyophilization and storage conditions on the stability, antibacterial activity, cytotoxicity, and physicochemical properties of the formulated novel plant-based CMPLGA nanoparticles. The potential payload at the site of infection (ileum) was determined using a nano-dissolution (ND) in a simulated intestinal fluid, while the possibility of polymer leakage was determined in deionized water over 24 weeks. The prolonged storage at 25 and 4 ºC did not significantly reduce the antibacterial activity of the plant-based CMPLGA nanoparticle against *Salmonella* and *E. coli* over 24 weeks. Moreover, the use of CMPLGA nanoparticles enhanced the antibacterial activity of *T. sericea, L. camara, G. tenax,* and *C. tridens* extracts. The plant-based CMPLGA nanoparticle increased in size after lyophilization due to aggregation, while the zeta potential and polydispersity of the nanoparticles did not change significantly (zeta potential ≤ 7 mV and polydispersity index of ≤ 0.6), which indicates the nanoparticles' stability. After 24 weeks of storage, the nanoparticles showed only minimal cytotoxicity to NIH 3T3 cells with IC50 values ≥60 µg/mL.

In conclusion, the medicinal plants used in northern Namibia have the potential as sources of complementary and alternative antibacterial compounds for MDR *Salmonella* and *E. coli*. Additionally, the lyophilized plant-based-CMPLGA nanoparticles could improve the release properties of the antibacterial extracts at a pH of 1.2 (stomach pH) and 7.4 (ileum pH) and maintain the physicochemical. Moreover, the storage temperature of lyophilized plant-based-CMPLGA nanoparticles does not affect the antibacterial and cytotoxicity

properties of antibacterial extracts. Hence, the plant-based CMPLGA nanoparticles formulated in this study should be used as a basis and evidence for the application of plant-based nanomedicine to fight MDR diarrheal bacteria.

It is however recommended that more studies documenting and validating the potential ethnomedicinal knowledge of plants in treating gastroenteritis should be conducted in other parts of Namibia to document plants with the future potential sources of medicine for gastrointestinal conditions. The phytochemical analysis using in-depth methods such as High-Performance Liquid Chromatography (HPLC), Mass spectroscopy/ Gas chromatography should be used to quantify all the phytochemical compounds in the studied extracts. The efficacy of the formulated plant-based CMPLGA nanoparticles should also be tested against other gastrointestinal bacteria species of *Campylobacter, Shigella*, and *Vibrio cholera*. Additionally, *in-vitro* studies using gastrointestinal cell lines such as Caco2 cell lines should also be determined to determine the effect of these formulations specifically on gastrointestinal tract cells. In addition, the formulated nanoparticles should be evaluated in an *in vivo* model to determine whether there is consistency in results regarding the efficacy, toxicology, and biophysical properties of the nanoparticles, as well as to determine the effective dosage of the nanoparticles. It is also recommended that different ratios of chitosan which are known to have mucoadhesive properties be used to see how this may affect the toxicology, efficacy, and biophysical properties of the plant-based CMPLGA nanoparticles. Lastly, it is recommended that the modes of action for the formulated plant-based CMPLGA nanoparticles should be determined as this will determine whether the nanoparticles are bacteriostatic or bactericidal.

CHAPTER 8: CONTRIBUTION TO NEW KNOWLEDGE

This study has several new contributions to the scientific body of knowledge as explained below:

1. Documenting the ethnomedicinal uses of medicinal plants in Iikokola village, Omusati region, Namibia, especially for plants such as *L. camara, S. capense, O. kilimands, P. camestris, C. tridens,* and *G. tenax* that are used to treat gastrointestinal conditions.
2. Providing the antigram report for using clinical isolates of *Salmonella* and *E. coli* from Namibian pediatrics.
3. Fabrication of pH-dependent CMPLGA nanoparticles containing *T. sericea, L. camara, G. tenax,* and *C. tridens* for their improved delivery within the ileum. This offers the advantage of protecting the extract from the acid pH of the stomach while increasing the release within the ileum. This may also increase the bioavailability and biocompatibility of the fabricated nanoparticles.
4. The Physico-chemical characterization of the effects of storage lyophilization, time, and temperature on the in vitro biophysical properties of plant-based CMPLGA nanoparticles,
5. Value addition to the field of medicinal IK by integrating FDA-approved nanotechnology through PLGA and Chitosan polymers, to improve the efficacy, safety, and pharmacological properties of medicinal plant extracts for gastrointestinal uses.

6. The findings of this study create bases for future research aiming at developing targeted nanoparticles for gastroenteritis and other gastrointestinal conditions.

CHAPTER 9: APPENDICES

Appendix A: Ethical Clearance

UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOS-0039 Date: 04 March 2022

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project: DESIGNING AND CHARACTERIZING THE PLANT-BASED CHITOSAN MODIFIED PLGA NANOPARTICLES FOR SALMONELLA AND E. COLI INDUCED GASTROENTERITIS

Student: SHATRI ALBERTINA

Student Number: 200919563

Supervisor(s): PROF. DAVIS MUMBENGEGWI; DR. YOLANDY LEMMER

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

Appendix B: Plant collection permits

MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 2221/2017
Valid from 9 November 2016 to 31 October 2017

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

Name: **Ms C.I. Du Preez**
Address: **Multidisciplinary Research Centre**
University of Namibia
Private Bag 13301
Windhoek
Namibia
Coworkers: **Dr. D. Mumbengegwi, Ms. K. Kaitjizemine, Dr. H. Winschiers, Mr. G. Katjipure, Mr. C. Stanley, Mr. M. Chamunorwa, Mr. M. Shirungu, Mr. W. Embashu and Dr. A. Cheikhyousseff**

National survey on documentation of Indigenous Knowledge System (specifically on medicine, food and beverage) in Hardap, Omusati, Erongo and Omaheke Regions, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way.

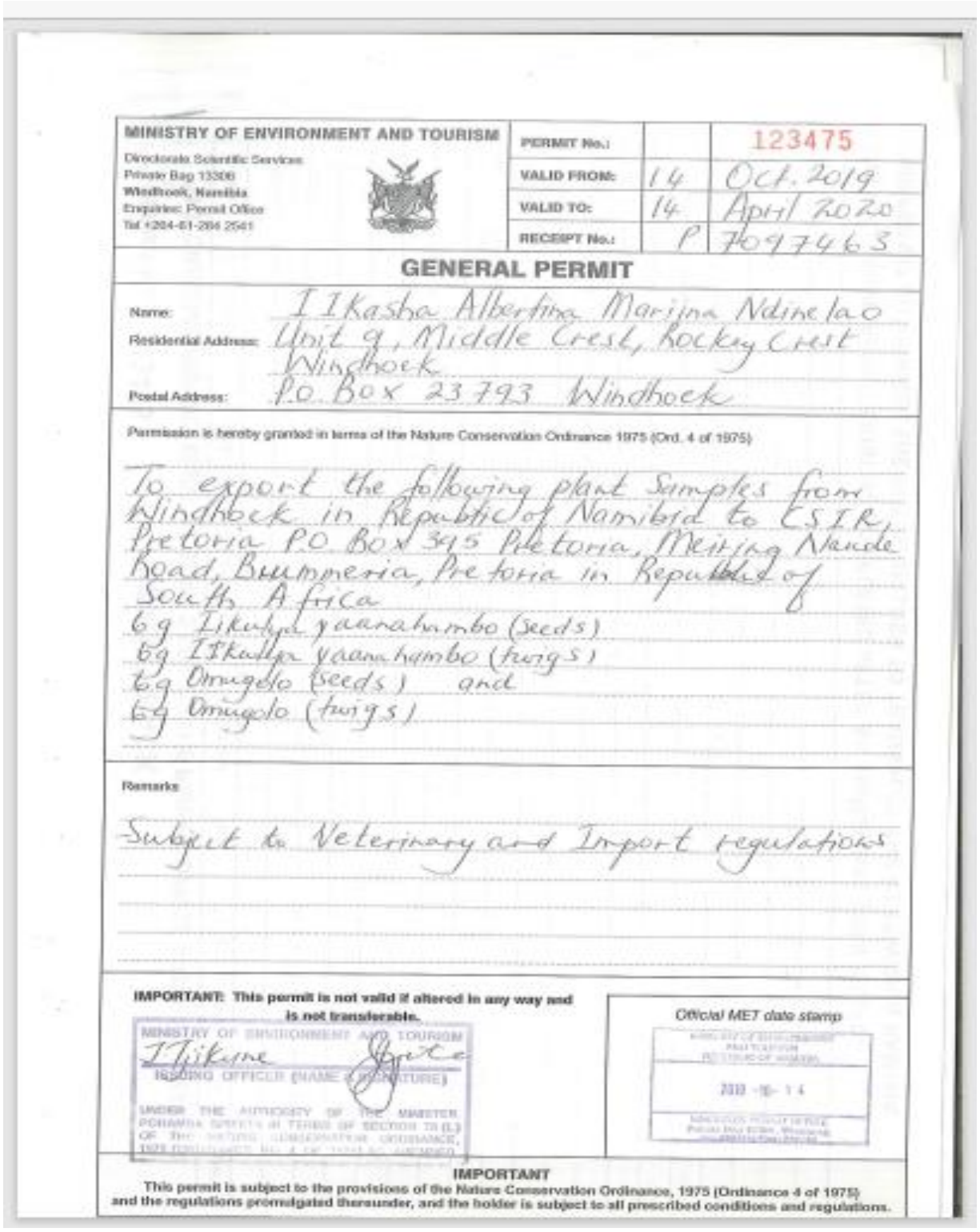
MINISTRY OF ENVIRONMENT AND TOURISM
REPUBLIC OF NAMIBIA
16 MAR 2017
WINDHOEK
Private Bag 13306, Windhoek
Tel: 2842111 • Fax: 258861

Authorising Officer

IMPORTANT
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Conservation Scientist, email ilta.matheus@met.gov.na
Private Bag 13306, Windhoek, Namibia

Appendix C: Plant Export permit



MINISTRY OF ENVIRONMENT AND TOURISM

Directorate Scientific Services
Private Bag 13306
Windhoek, Namibia
Enquiries: Permit Office
Tel +264-61-284 2541

PERMIT No.:		123475
VALID FROM:	14	Oct. 2019
VALID TO:	14	April 2020
RECEIPT No.:	P	7097463

GENERAL PERMIT

Name: I Ikasha Albertina Marijna Ndinelao
Residential Address: Unit 9, Middle Crest, Rocky Crest Windhoek
Postal Address: P.O. Box 23793 Windhoek

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)

To export the following plant samples from Windhoek in Republic of Namibia to CSIR, Pretoria P.O Box 395 Pretoria, Meiring Naude Road, Brummeria, Pretoria in Republic of South Africa
6g Ikukya yaanahambo (seeds)
6g Ikukya yaanahambo (twigs)
6g Omugolo (seeds) and
6g Omugolo (twigs)

Remarks

Subject to Veterinary and Import regulations

IMPORTANT: This permit is not valid if altered in any way and is not transferable.

MINISTRY OF ENVIRONMENT AND TOURISM
I Ijikuna
ISSUING OFFICER (NAME & SIGNATURE)
UNDER THE AUTHORITY OF THE MINISTER
PERMITS EFFECTS IN TERMS OF SECTION 78 (1)
OF THE NATURE CONSERVATION ORDINANCE,
1975 (ORDINANCE NO. 4 OF 1975) AS AMENDED

Official MET date stamp

2019 -10- 14

IMPORTANT

This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all prescribed conditions and regulations.

Appendix D: Informed consent

INFORMED CONSENT FOR QUALITATIVE STUDIES

Informed Consent for Ethno medicinal knowledge holders in Iikokola village who we are inviting to participate in a survey about Indigenous knowledge of plants used to treat diarrheal diseases (Acute gastroenteritis).

Name of Principal Investigator:	Albertina Shatri
Name of Sponsor:	University of Namibia

This Informed Consent Form has two parts:

- **Information Sheet (this section, to share information about the study with you)**
- **Certificate of Consent (for signatures if you choose to participate)**

You will be given a copy of the full Informed Consent Form.

PART I: INFORMATION SHEET

My Name is Albertina Shatri. I am a student at the University of Namibia (UNAM), doing my doctoral (Ph.D.) degree on a project that is in collaboration between the Multidisciplinary Research Centre and the Faculty of Health Sciences (School of Medicine).

We are conducting a study on Indigenous knowledge of plants used to treat diarrheal diseases (acute gastroenteritis). We would like to ask you questions relating to this knowledge and to identify and collect the plants to analyze them in the laboratory for antidiarrheal properties. The interview will be conducted individually and will take a maximum of 15 minutes. You are being invited to take part in this research because we have noticed that the knowledge of traditional health care practitioners in Iikokola village is not documented and we feel that your experience may help inform the next generation on the potential sources of plant-based medicine for treating gastroenteritis in Namibia. The findings of this study will be shared with you and you will be consulted before any further studies are conducted. The results of the study may be published. The main aim of the study is to validate plants used to treat diarrhea and to develop a treatment based on medicinal plants for children under the age of five and we would like your consent to participate in this

study. Your sharing of this information is voluntary and your choosing to participate or not will not have any bearing on your job or job-related evaluations. The information from this study will be treated confidentially and will be used for research purposes only. There are no risks associated with this research

PART II: CERTIFICATE OF CONSENT

I …… have been invited to participate in research about gastroenteritis and local health practices.

(This section is mandatory.)

I confirm that this study and its objectives have been explained to me. I fully understand the nature and purpose of this study and I have had the opportunity to ask questions about it any questions I have been asked, have been answered to my satisfaction. I consent voluntarily to be a participant in this study

……. ……

Name of Participant (print) Signature of Participant

……

Date (day/month/year)

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

…….

Name of Witness (print)

……

Signature of Witness

……

Date (day/month/year)

Statement by the Researcher/Person taking Consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. The purpose of the study.
2. The information required from participants.
3. The risks and benefits of the study
4. Confidentiality
5. Informed written consent

I confirm that the participant was allowed to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

……. ……

Name of Researcher/Person taking Consent (print) Signature

……

Date (day/month/year)

Appendix E: Questionnaire

QUESTIONNAIRE

Dear Participant

1. My name is Albertina Shatri student number 200919563 I am studying towards a Doctorate (Ph.D.) degree at the University of Namibia (UNAM), and I am surveying Indigenous knowledge of plants used to treat diarrheal diseases (Acute gastroenteritis). I have selected you to participate in my study because you belong to the group of people I want to include in my research. I would therefore like to invite you to complete this questionnaire.
2. The research I am conducting has been approved by the UNAM Research Ethics Committee. I would appreciate it very much if you would complete this questionnaire, and I would like to assure you of the following:
    a. You do not have to fill in this questionnaire if you do not want to.
    b. You can stop filling in the questionnaire and stop participating at any time if you want to, and there will be no negative consequences for you.
    c. Your participation is completely anonymous. This means that, even if I ask for information that might identify you or if I know you, I am not allowed to make your identity known to anyone. When I report on my questionnaires' data and results, I will not mention any personal information about participants that might identify them.
    d. All completed questionnaires and data will be stored in a safe and secure place, and only authorized University officials, my supervisor, and I will have access to it. After five years, all the questionnaires and data will be destroyed in an environmentally friendly way.
3. If you have any questions about this questionnaire, or if you do not understand anything, please feel free to ask me, and I will be happy to explain it to you.
4. If you want to know more about the research I am doing, please feel free to ask me, and I will be happy to tell you more.
5. It should take about 15 minutes for you to complete the questionnaire.
6. You can reach me on my cell phone at 0812609607, or send an e-mail to aiikasha@unam.na.
7. If you want more information about the study *you can contact (**Albertina Shatri**) or the Centre for Research & Services at kmbulu@unam.na* if *you have any further queries about the study or* **if** *you have any concerns or complaints that have not been adequately addressed by the research team.*
8. Thank you very much for your willingness to participate in this research!

Please detach this page and keep it.

Please turn over to start filling in the questionnaire.

SECTION A: Participant's information

Name and Surname	
ID number	
Date of birth	
Region	
Village	
GPS coordinate	
Contact details	
Investigator	
Date	

Section B: Source of knowledge

Question	**Response**
How and from who did you get this knowledge?	
When did you start using medicinal plants?	
Are you teaching anyone this knowledge?	
If yes, why? If not why?	
Do you think medicinal plants can cure diarrhea?	
If yes/ No, why?	
What factors do you think can influence the use of traditional medicine?	
Do you also use allopathic medicine?	
Does religion, economic status, lifestyle change, and access to modern medicine influence the use of traditional medicine?	
If yes, why, If no, why?	

Section C: Level of knowledge on diarrhea (The Disease)

Question	Response
Please tell us what you know about diarrhea	
What do you think causes diarrhea?	
Do you treat diarrhea?	
How do you treat diarrhea?	
Do you think medicinal plants can cure diarrhea?	
If yes/ No, why?	

Section D: Plants used to treat/ manage diarrhea (Expand information using the table in section E)

Question	Response
What plant do you use to treat/manage diarrhea?	
Is it used in combination with other plants? If Yes why?	
Which part of the plant is used to treat diarrhea?	
How is the medicine prepared?	
How is it administered?	
How long does it take for the treatment to work?	
When do you harvest the plants?	
Are there ways in which you conserve/ preserve the plant?	
Any other uses apart from diarrhea treatment?	

Section E: Information on specific plants used to treat diarrhea

Plant local name	Scientific name	Diarrheal uses	Part used	Preparation method	Dosage	Recovery period	Age limit	Any other uses
1.								
2.								
3.								
4.								
5.								
6.								

Section F: Specific plants of interest

Do you use the following plants?

Plant local name	Scientific name	Diarrheal uses	Part used	Preparation method	Dosage	Recovery period	Age limit	Any other uses
1. Omupopo	*Albizia anthermintica*							
2. Oshishegele	*Grewia tenax*							
3. Omutuutuu	*Piliostigma thonningii*							
4. Oshipeke	*Ximenia Americana*							
5. Omugolo	*Terminalia sericea*							

6. Ohama	*Terminalia Prunioides*							
7. Omusheshete	*Ziziphus mucronate*							

Appendix F: Plant Collection Form

NBRI: DATA COLLECTION FORM

Collect.No. ..... Date .....
Collector/s ..... GPS ..... S
Locality (please describe properly, not just GPS) ..... E
..... Grid .....
..... Altitude .....
Aspect ..... Slope ..... Exposure .....
Photo no's ..... Voucher for .....

DESCRIPTION OF PLANT Please give as much information as possible
Species ..... Perennial/Annual
Habit (e.g.shrub) ..... Height ..... cm
Occurrence (e.g.common) .....
Indigenous names and languages .....
.....
Flower/Inflorescence (colour,shape,size,smell,texture) .....
.....
Leaves (simple/compund,margin,shape,colour,texture,stipules) .....
.....
Stems and bark (colour,texture,habit - e.g. erect stem with yellow papery peeling bark - presence or absence and smell of sap or latex - e.g. milky latex with unpleasant smell) .....
.....
Fruits and seed (presence/absence,shape,colour,edibility and taste if edible, single or clustered,maturity) .....
.....
Roots and underground organs (shape,texture,colour,size) .....
.....
Other (e.g.: uses,ecology) .....
.....

Habitat			Vegetation	Substrate	Soil type	Lithology	Moisture	Biotic effect
cliff face	plain	lake/pond	desert	soil	sand		well-drained	road/rail side
mountain/ hillslope	plateau	marsh/ swamp/ wetland	shrubland open/closed	stony/rocky soil	sandy loam	mica schist	moist/damp	cultivated land
mountain/ hill peak	valley	river/stream	forest	gravel	loam	dolomite	seasonally waterlogged	abandoned land
talus/scree	donga/ gulley/ ditch	river/stream bank	grassland	bare rock	loamy clay	granite	permanently waterlogged	heavily grazed
					gravel			
dune slope	depression/ pan	seepage area	karroo	water	clay	lime	free standing water	garden
dune crest	dam	waterfall/ rapids	thornbush savanna	roots	calcrete	quartzite	running water	plantation
interdunal street	dry river/ stream bed	unknown		termite mound	black turf	other	mist/fog	recently burnt
ravine/kloof/ gorge	floodplain			other	humus rich		tidal	disturbed – other
ridge	estuary/ lagoon				salt/brack			other

Appendix G: NBRI plant identification report

Ministry of Agriculture, Water and Forestry

National Herbarium of Namibia (WIND)

Identification Report

Report No.: 2017/390

25 July 2017

Collector/s: Du Preez, I

Address: Unviersity of Namibia
P/Bag 13301
Pionierspark
Windhoek

Number	ID cat.	Identification
s.n	1	Marsdenia macrantha (Klotzsch) Schltr.
s.n	4	
BRL 20	1	Hoodia gordonii (Masson) Sweet ex Decne.
T 02-05	3	Hoodia sp.
BRL 40	1	Terminalia sericea Burch. ex DC.
BRL 43	1	Adenium boehmianum Schinz
BRL 51	1	Terminalia sericea Burch. ex DC.
BRL 30	1	Chorchorus tridens L.
BRL 33	1	Grewia tenax (Forssk.) Fiori
BRL 23	1	Zygophyllum decumbens Delile var. decumbens
BRL 35	1	Terminalia prunioides M.A.Lawson
BRL 36	1	Drimia sanguinea (Schinz) Jessop
T 03-04	2	Tulbaghia violacea
T 01-05	3	Carpbrotus sp.
BRL 38	1	Asparagus exuvialis Burch.
T 02-04	3	Aloe sp. Baker

BRL 38	1	Asparagus exuvialis Burch.
T 02-04	3	Aloe sp. Baker
BRL 21	3	Aloe hereroensis Engl.
BRL 25	1	Aloe zebrina Baker
T 03-03	1	Bulbine frutescens (L.) Willd.
BRL 27	1	Bulbine frutescens (L.) Willd.
BRL 26	1	Hermannia fruticulosa K.Schum.
BRL 28	1	Sarcocaulon salmoniflorum Moffett
BRL 30	3	Psidium sp.
BRL 36	2	Moringa ovalifolia Dinter & A.Berger
BRL 37	1	Ziziphus mucronata Willd.
BRL 42	1	Pollichia campestris Aiton
T 01-06	1	Punica granatum L.
T 01-07	2	Petroselinum crispum (Mill.) Fuss
T 01-09	2	Petroselium crispum (Mill.) Fuss
T 01-10	2	Petroselium crispum (Mill.) Fuss
T 01-12	2	Myrothamnus flabellifolius Welw.
T 02-03 (a)	1	Blepharis obmitrata C.B.Clarke

Identification categories: 1. Certain identification 2. Closest to 3. Certain to genus only 4. Unable to identify

Private Bag 13184, Windhoek Tel: +264 - 61 - 202 - 2021 Fax: +264 - 61 - 259 - 153 e-mail: Frances.Chase@mawf.gov.na 1

Number	ID cat.	Identification
AI 01	1	Ziziphus mucronata Willd.
AI 06	1	Solanum lichtensteinii Willd.
AI 05	1	Sesamum capense Burm.f.
AI 07	1	Catharanthus roseus (L.) G.Don
FD 11	3	Ipomoea
FD 12	1	Pergularia daemia (Forssk.) Chiov.
FD 20	1	Gomphocarpus fruticosus (L.) W.T.Aiton
KS 01	3	Diospyros lycioides Desf.
FD 10	1	Polydora poskeana (Vatke & Hildebr.) H.Rob.
FD 13	1	Dicoma tomentosa Cass.
FD 15	1	Emilia schinzii (O.Hoffm.) Cron
AI 04	1	Hibiscus dongolensis Delile
AI 03	1	Terminalia prunioides M.A.Lawson
FD 14	1	Gomphocarpus fruticosus (L.) W.T.Aiton
TONI 2	1	Diospyros lycioides Desf.
TONI 1	1	Dichapetalum cymosum (Hook.) Engl.
AI 08	1	Asparagus exuvialis Burch.
FD 09	1	Ipomoea obscura (L.) Ker Gawl.
AI 02	1	Boscia albitrunca (Burch.) Gilg & Gilg-Ben.
NE 001	1	Adenolobus garipensis (E.Mey.) Torre & Hillc.

Number	ID cat.	Identification
T 02-03 (b)	1	Dicoma schinzii O.Hoffm.
T 02-06	1	Thamnosma africana Engl.
T 02-09	1	Ziziphus mucronata Willd.
T 03-01	2	Nymania capensis (Thunb.) Lindb.
T 01-08	3	Amaranthus sp. Thell.
T 01-07	1	Senna italica Mill.
BRL 41	1	Ximenia americana L. var. americana
BRL 34	1	Albizia anthelmintica (A. Rich.) Brongn.
BRL 52	1	Acacia erioloba E.Mey.
T 02-01	1	Calophractes alexandri D.Don
BRL 44	1	Colophospermum mopane (J.Kirk ex Benth.) J.Kirk ex J.Léonard
BRL 39	1	Colophospermum mopane (J.Kirk ex Benth.) J.Kirk ex J.Léonard
BRL 24	1	Harpagophytum procumbens (Burch.) DC. ex Meisn. var. procumbens
BRL 20	1	Acacia erioloba E.Mey.
T 02-08	4	
T 03-02	1	Kleinia longiflora DC.
BRL 29	1	Leonotis ocymifolia (Burm.f.) Iwarsson var. schinzii
T 1-13	4	
T 01-11	1	Rosmarinus officinalis
T 01-03	3	Mentha sp.
T 01-04	1	Ocimum americanum L.
T 01-02	2	Ocimum filamentosum Forssk.

Appendix H: Linear regression curves and equations

Linear regression curve for total quantification of phenols and flavonoids CHAPTER 3

Total phenolic content

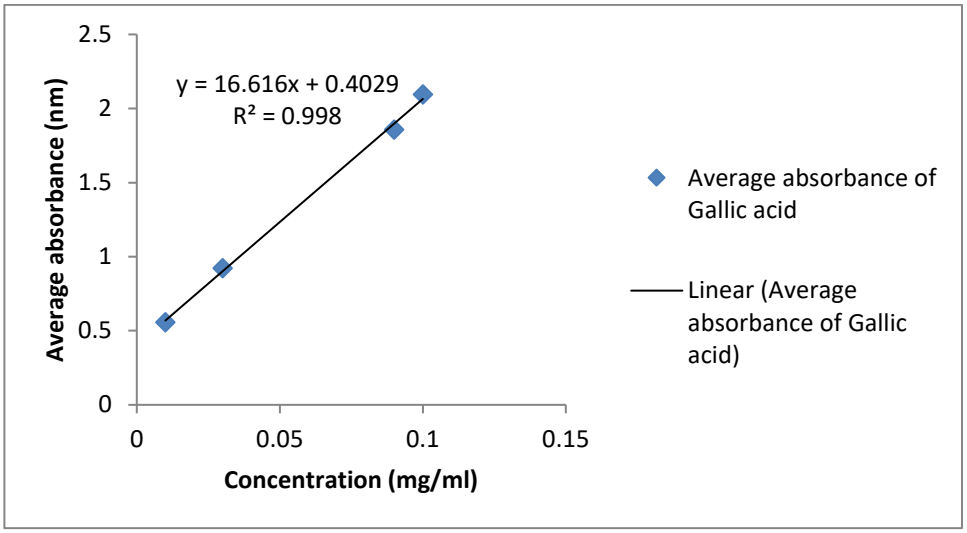


Figure 1: Linear data of Gallic acid

Total flavonoids content

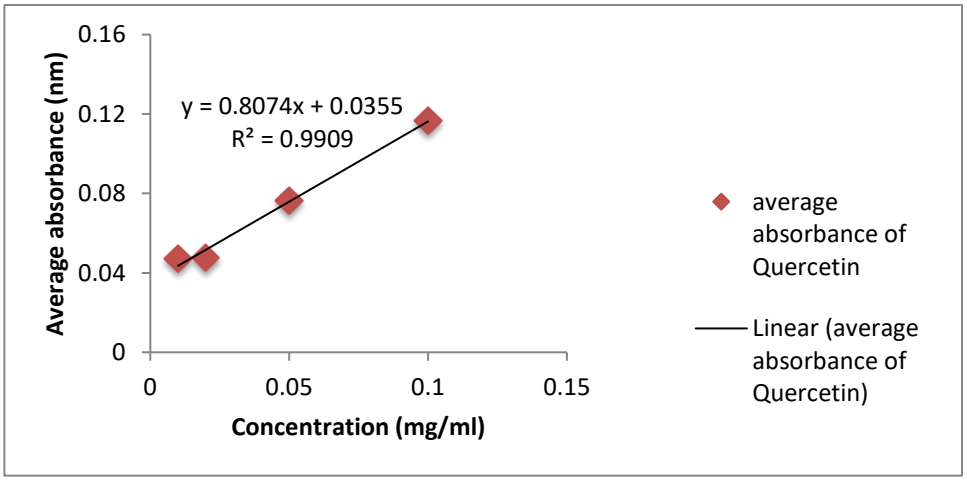


Figure 2: Linear data of quercetin

Linear regression for Encapsulation efficacy and invitro release chapter 5

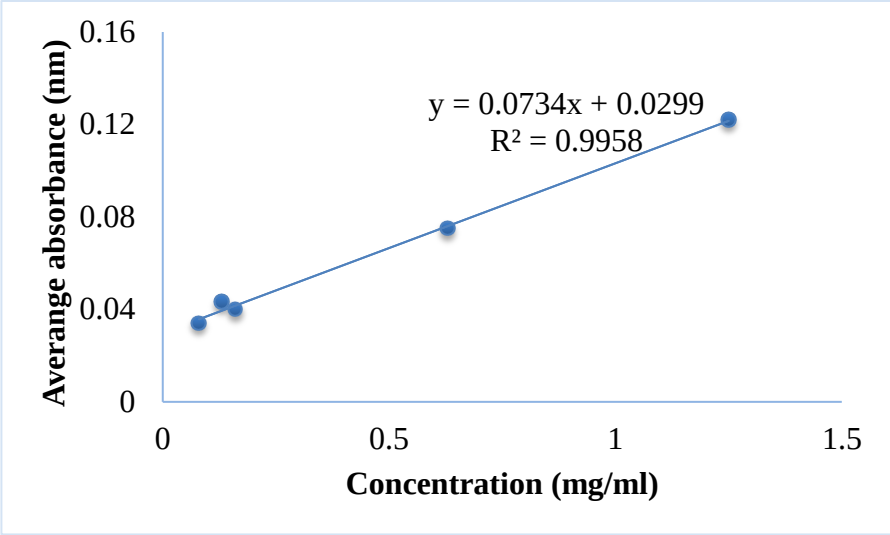


Figure 3: Calibration curve of *C. tridens* extract for the determination of EE% and *in-vitro* release profile

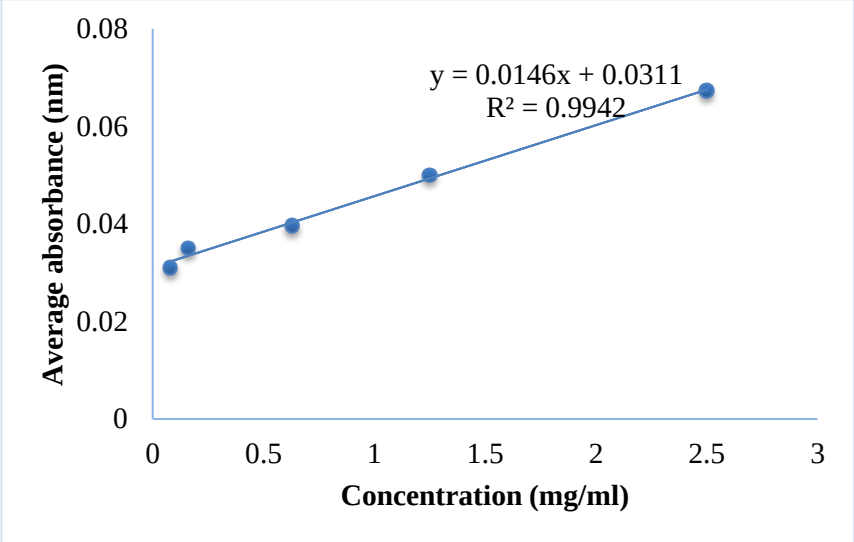


Figure 4: Calibration curve of *L. camara* twig extract for the determination of EE% and *in-vitro* release profile

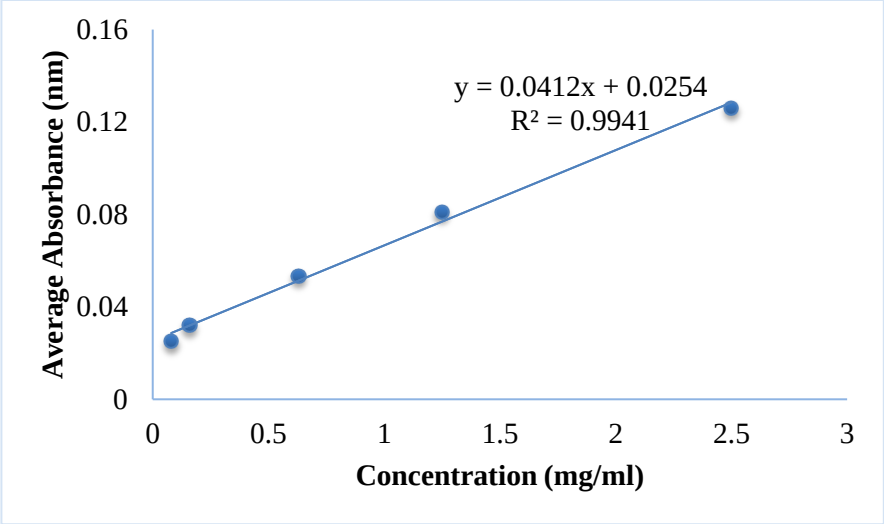


Figure 5: Calibration curve of *L. camara* seeds extract for the determination of EE% and *in-vitro* release profile

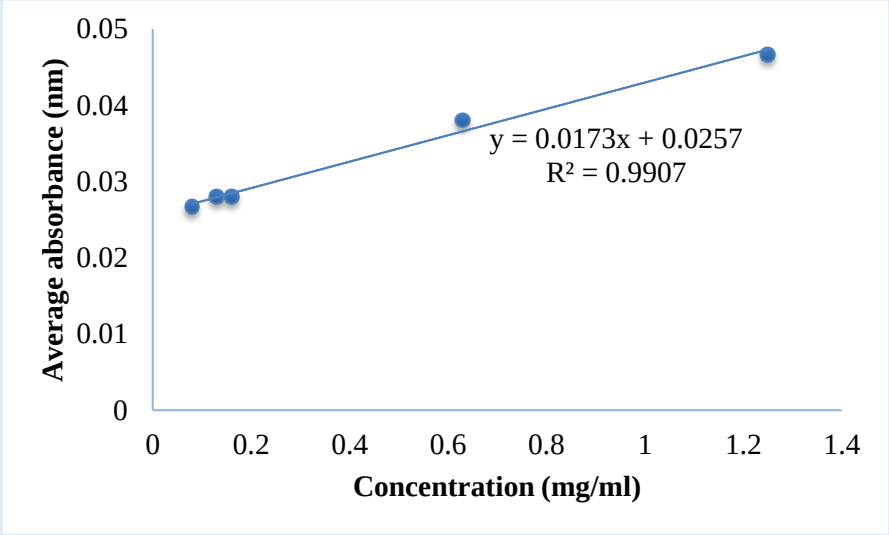


Figure 6: Calibration curve of *G. tenax* for the determination of EE% and *in-vitro* release profile

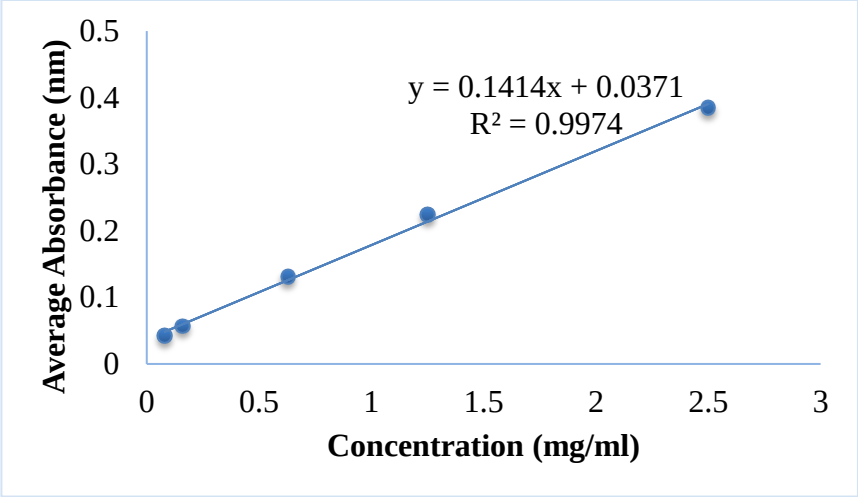


Figure 7: Calibration curve of *T. sericea* for the determination of EE% and *in-vitro* release profile

Equations and calculations of in vitro release at 1.2 and 7.4 pH

The equations used are in figure 3-7

Y=mx=c

Extract concentration (x)= (Average Absorbance-Intercept)/Slope

Extract amount= (Extract concentration X Batch volume X Dilution factor)/1000

Cumulative extract release= (Extract amount/Batch strength) X 100

Appendix I: Detailed protocols for preparation of solvent systems and chromogenic reagents (CHAPTER 3) as well as simulation preparation for *in vitro* release (CHAPTER 5)

A. Detailed method for phytochemical screening (CHAPTER 3)

Freshly prepared organic and aqueous plant extracts were screened for the presence of flavonoids, alkaloids, tannins, steroids, and coumarins by Thin layer Chromatography (TLC). Plant extracts (organic and aqueous) were prepared by dissolving 32 mg plant extract in 4 ml 90 % methanol for organic extracts and 32 mg plant extracts in 4 ml distilled water for aqueous extracts. The extracts were then vortexed for 5 minutes.

The presence of coumarins, alkaloids, tannins, steroids, flavonoids, and triterpenoids in the plant extracts (organic and aqueous extracts) of *T. sericea* (twigs and seeds) *L. camara* (twigs and seeds), *G. tenax* (roots and twigs), *Corchorus tridens* (whole plant), and *A. anthelmintica* (roots and twigs) was determined by using methods adapted from Harborne (1998). The preparation of solvents used in the screening of each bioactive compound is described below:

Screening for Alkaloids

Chloroform: ethanol in a ratio of 9:1 was used as the solvent system. Dragendorff reagent was used as a chromogenic reagent for screening alkaloids in different plant extracts. Quinine was used as a positive control. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. The plant extracts were applied in a sequential application while allowing the drying of the spots. The spots were made 2 cm

from the left to the right edge of the TLC plate with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Chloroform: Ethanol (9:1) solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with Dragendorff reagent, the plates were viewed under UV light at 360 nm. The presence of the orange and blue fluorescence at 360nm represented the presence of alkaloids. The Rf value for each spot was calculated.

Screening for Flavonoids

Butanol: acetic acid: Water in a ratio of 4:1:5 was used as the solvent system and 10% Antimony chloride in chloroform was used as a chromogenic reagent. Quercetin was used as a positive control. A 1 cm line was measured and drawn with a soft pencil on a TLC plate from the bottom of the TLC plate. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. The spots were made 2 cm from the left to the right edge of the TLC plate with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Butanol: acetic acid: Water in a ratio of 4:1:5 solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent, the plates were viewed under UV light at 360 nm and examined for the presence of yellow-orange, and blue fluorescence spots. The Rf value for each spot was calculated.

Screening for Saponins

Chloroform: methanol and water in a ratio of 7:3:1 was used as the solvent system and Vanillin, ethanol, and sulfuric acid were used to prepare the chromogenic reagent. Saponin was used as a positive control. A 1 cm line was drawn on a TLC plate using a soft pencil from the bottom of the TLC plate. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. The spots were made 2 cm from the left to the right edge of the TLC plate with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Vanillin, ethanol, and sulfuric acid solvent as depicted in figure 15. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent, the plates were viewed under UV light at 360 nm and examined for the presence of blue fluorescence. The Rf value for each spot was calculated.

Screening for Steroids

Chloroform: acetone was used as the solvent system. Two chromogenic reagents (A and B) were used. A: Phosphoric acid in water as the first chromogenic reagent. The TLC plates were heated in the oven at 120 °C for 20 minutes and then later sprayed with B: phosphoric acid in methanol as the second chromogenic reagent. B-sitosterol was used as a positive control. The presence of steroids was detected on the TLC plate by drawing a line 1 cm from the bottom of the TLC plate. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. Spots that are 2 cm apart were drawn with a soft pencil and it was on these spots where the plan extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Chloroform, and acetone. The mobile phase was allowed to run up the TLC plate and the

TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with, Chromogenic reagents A and B, the TLC plates were viewed under UV light at 360 nm and examined for the presence of dark spots upon heating at 120 °C. The Rf value for each spot was calculated.

Screening for Tannins

Exactly 1 % Potassium hydroxide in Methanol was used as the solvent system and 1 % ferric chloride in 50 % aqueous methanol was used as the chromogenic reagent. B-sitosterol was used as a positive control. A 1 cm line was drawn from the bottom of the TLC plate. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. Spots that are 2 cm apart were drawn on a TLC plate and it was on these spots where the extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of 1 % Potassium hydroxide in Methanol. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent, the TLC plates were viewed under UV light at 360 nm and examined for the presence of purple and orange fluorescence. The Rf value for each spot was calculated.

Screening for Coumarins

Chloroform was used as the solvent system and Copper sulfate, sodium citrate, and anhydrous sodium carbonate were used to prepare a chromogenic reagent. Coumarin was

used as a positive control. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. The spots were made 2 cm apart on a TLC plate and it was on these spots that the extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of chloroform. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank as depicted in figure 16. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent, the TLC plates were viewed under UV light at 360 nm and examined for the presence of blue long wavelength fluorescence. The Rf value for each spot was measured.

Screening for Triterpenoids

Hexane: ethyl acetate in a ratio of 17:3 was used as the solvent system and Liebermann reagent (acetic acid, sulfuric acid, and ethanol) was used as a chromogenic reagent. B-sitosterol was used as a positive control. A 1 cm line was drawn from the bottom of the TLC plate using a soft pencil. Approximately 10 µl of each plant extract was applied on a TLC plate using capillary tubes. The plant extracts were applied in a sequential application while allowing the drying of the spots. Spots that are 2 cm apart were drawn on a TLC plate and it was on these spots where the plant extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of mobile solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with, the chromogenic reagent, the TLC plates were

viewed under UV light at 360 nm and examined for the presence of blue, green, and yellow fluorescence as depicted in figure 17. The Rf value for each spot was calculated.

B. Preparation of simulated digestion mediums (CHAPTER 5)

Step 1

Simulated gastric and intestinal fluids were prepared for later step as follows:

A: The composition for simulated gastric fluid was 1.725 mL of KCl, 0.025 mL of KH2PO4, 3.125 mL of NaHCO3, 7.375mL of NaCl, 0.1 mL of MgCl2 · (H2O)6, and 0.125 mL of (NH4)2CO3.

B: The composition of simulated intestinal fluid was 1.725 mL of KCl, 0.025 mL of KH2PO4, 3.125 mL of NaHCO3, 7.375mL of NaCl, 0.1 mL of MgCl2 · (H2O)6, and 0.125 mL of (NH4)2CO3.

Step 2

A: For the simulated gastric medium, the medium was prepared by mixing 15 mL of simulated gastric

fluid, 0.01 mL of 0.3M CaCl2, and 1.8 mL of distilled water. And then the pH of the mixture was adjusted to 1.50±0.02 by the addition of 0.1 mol/L HCl solution.

B: For simulated intestinal medium the medium was prepared by mixing 11mL of simulated

intestinal fluid, 0.04 mL of 0.3M CaCl2, and water up to 20 mL. The pH of the mixture was adjusted to 7.0±0.2 with 0.1 mol/L NaHCO3 solutions. The above-prepared mediums were stored at 4 °C before subsequent experiments.

Appendix J: Pdi, Size, Morphology, and Zeta Potential of Formulated CMPLGA nanoparticles

A: Initial formulation (CHAPTER 5)

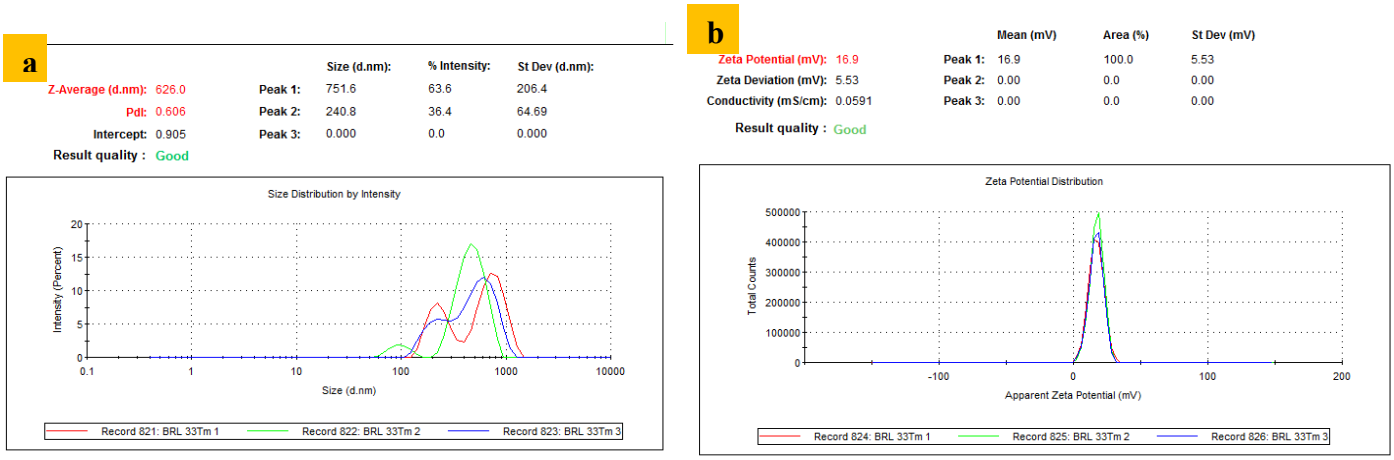


Figure 1: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(G) nanoparticles

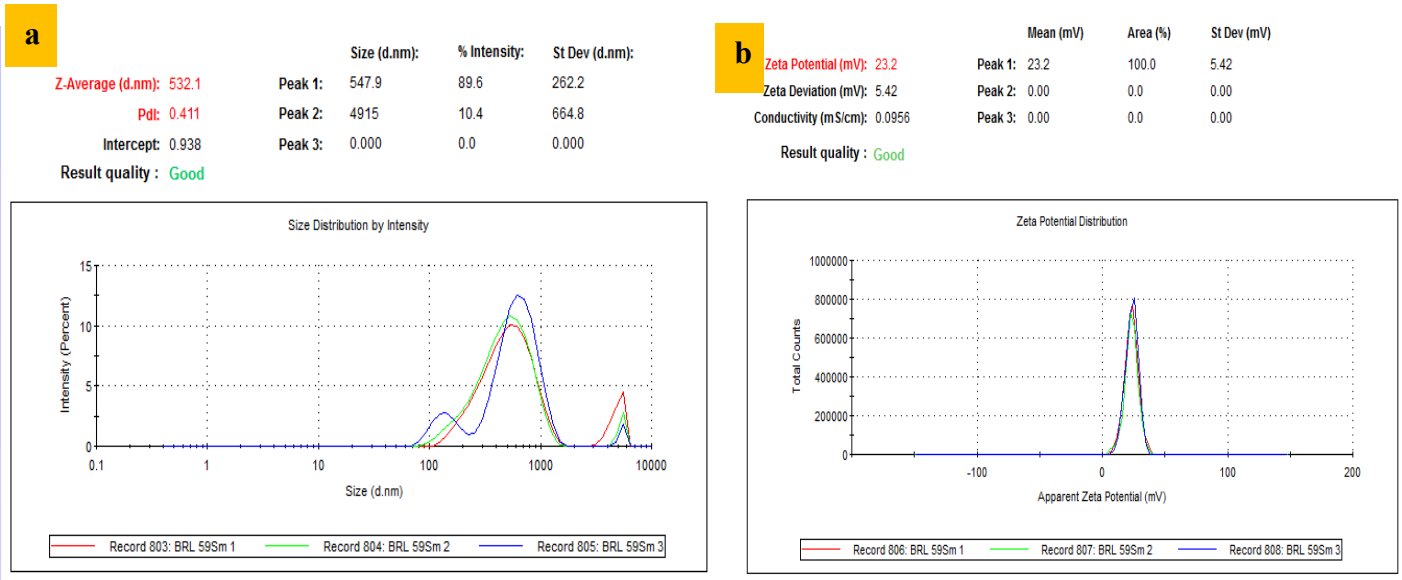


Figure 2: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(C) nanoparticles

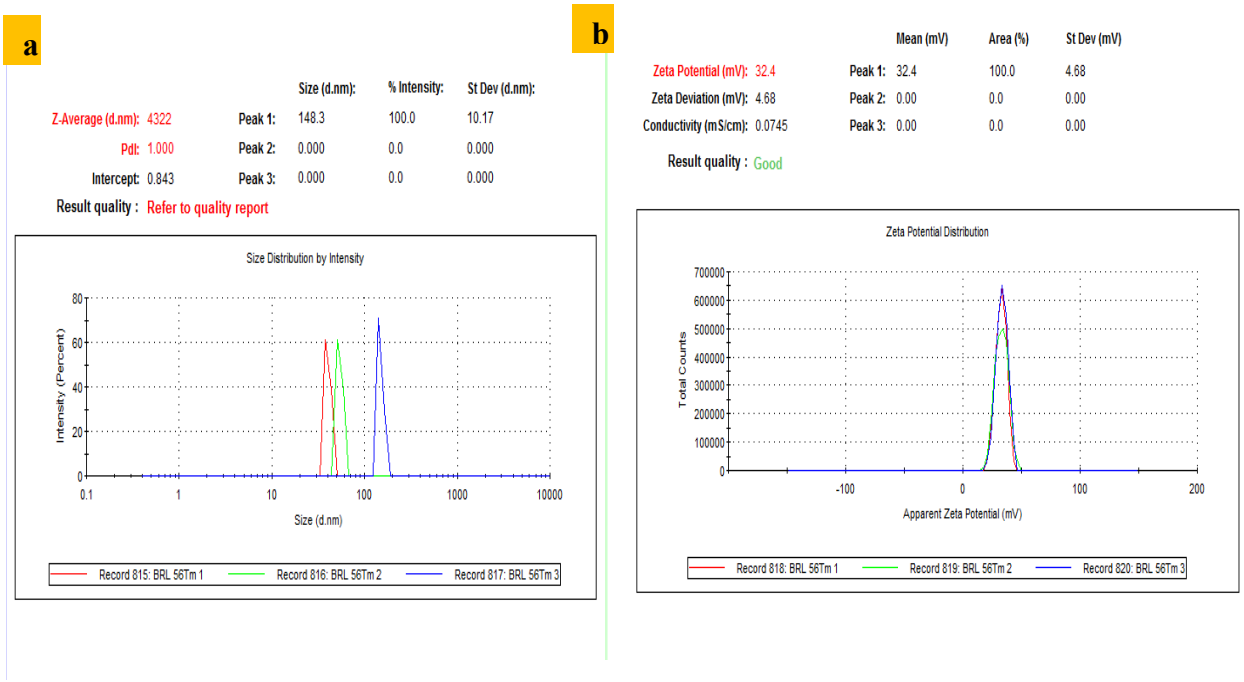


Figure 3: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(T) nanoparticles

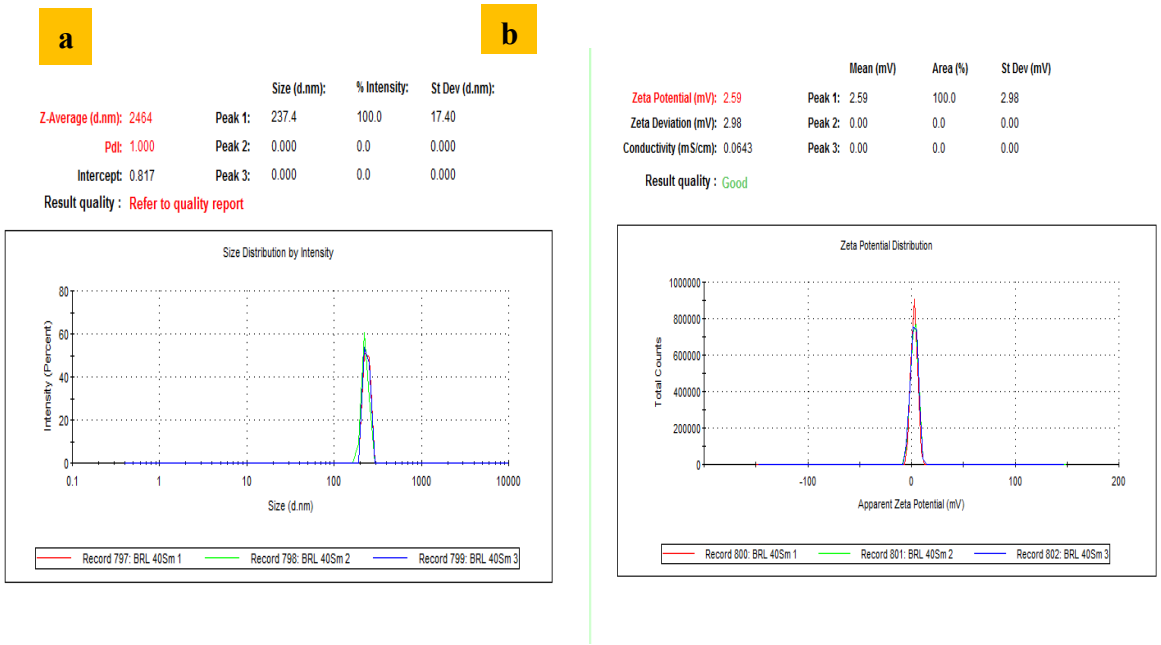


Figure 4: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(EE) nanoparticles

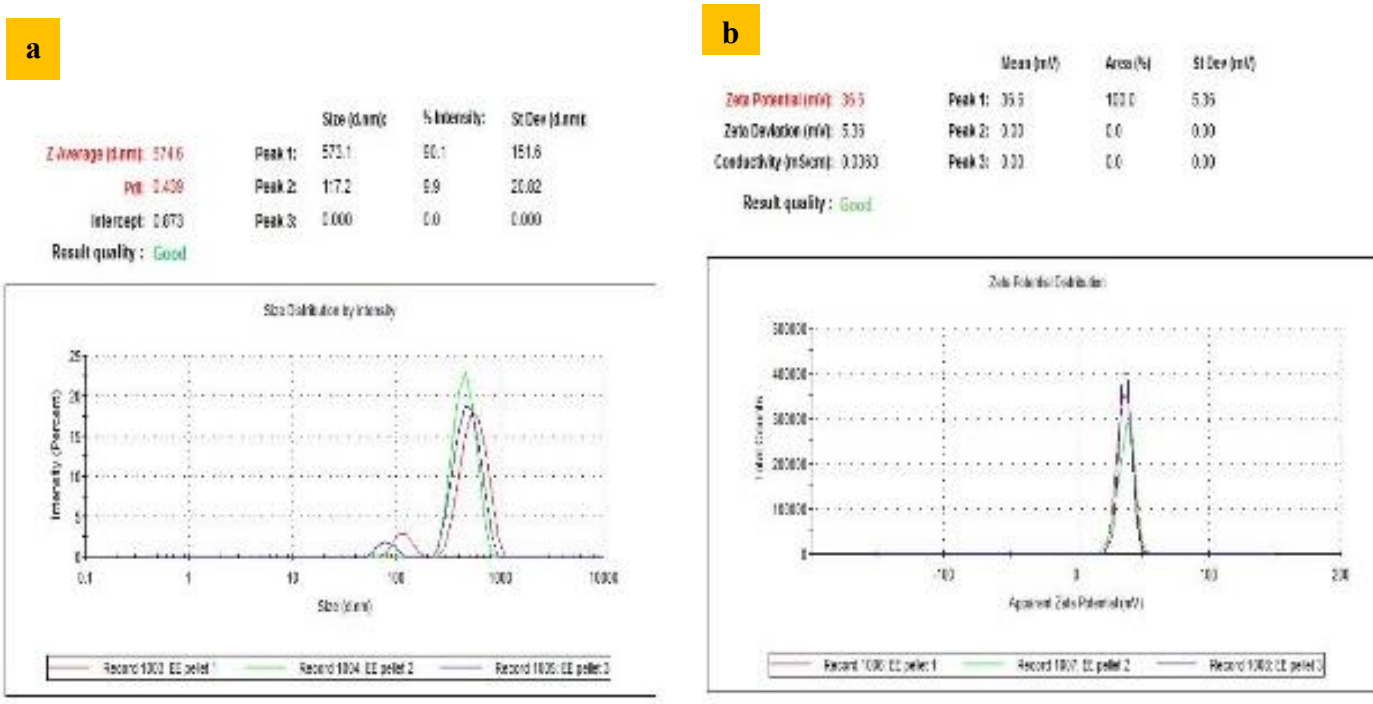


Figure 5: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(LS) nanoparticles

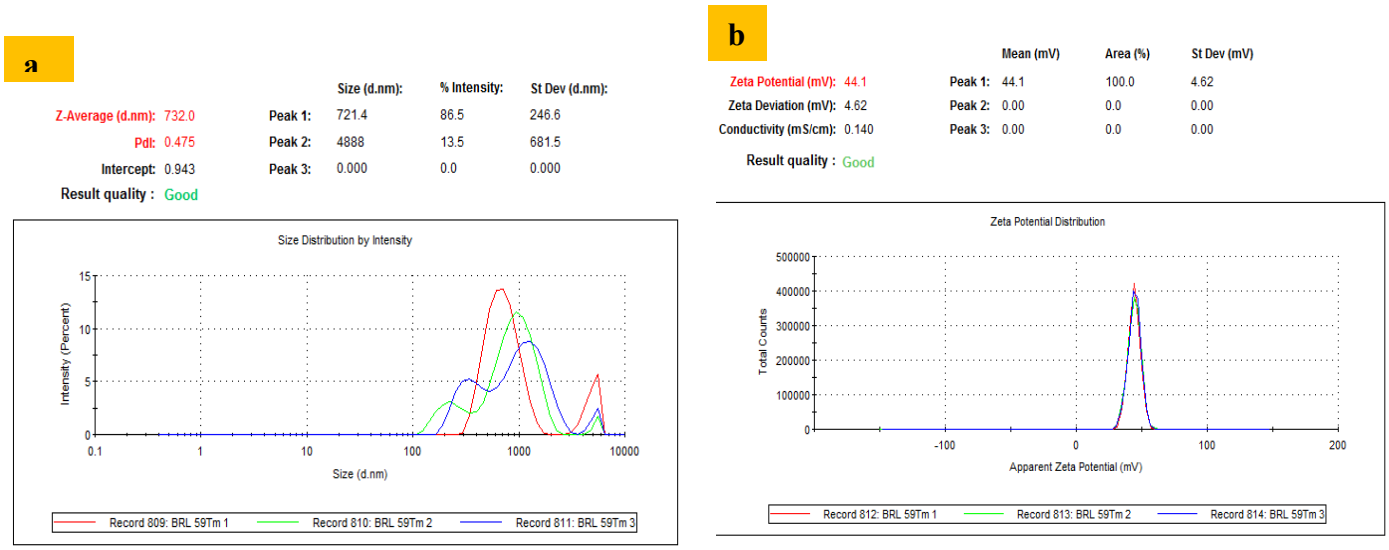


Figure 6: Particle size and particle size distribution (a), zeta potential (b) of CMPLGA(LT) nanoparticles

B: Formulation before freeze drying (CHAPTER 6)

Sample Name:	5-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	884	**Dispersant RI:**	1.330
Date and Time:	18 November 2019 14:07	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	295.5	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	8

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	4.89	**Peak 1:**	4.89	100.0	3.24
Zeta Deviation (mV):	3.24	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0190	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

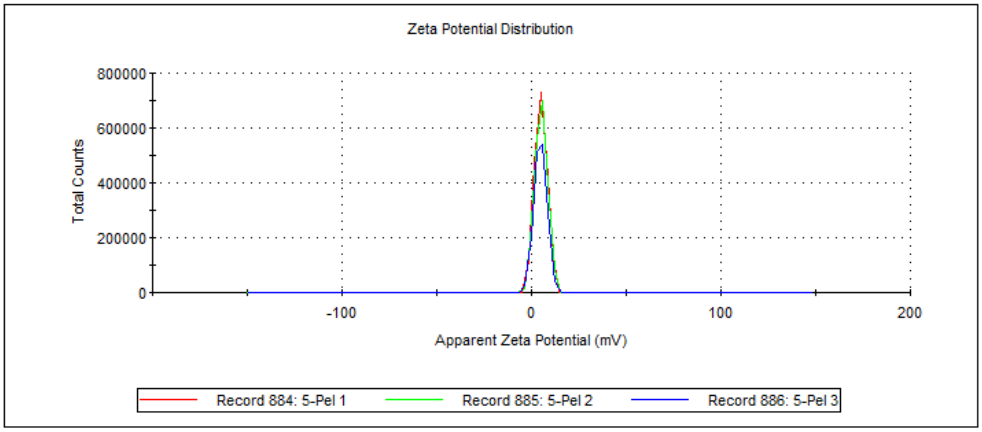


Figure 1: Particle zeta potential of CMPLGA(LT) pellet nanoparticles suspension

Sample Name:	5-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	881	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	18 November 2019 14:02

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	259.8	**Measurement Position (mm):**	0.85
Cell Description:	Disposable sizing cuvette	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	634.4	**Peak 1:**	862.8	99.1	472.8
PdI:	0.281	**Peak 2:**	56.89	0.6	14.65
Intercept:	0.951	**Peak 3:**	31.73	0.3	4.690
Result quality :	Good				

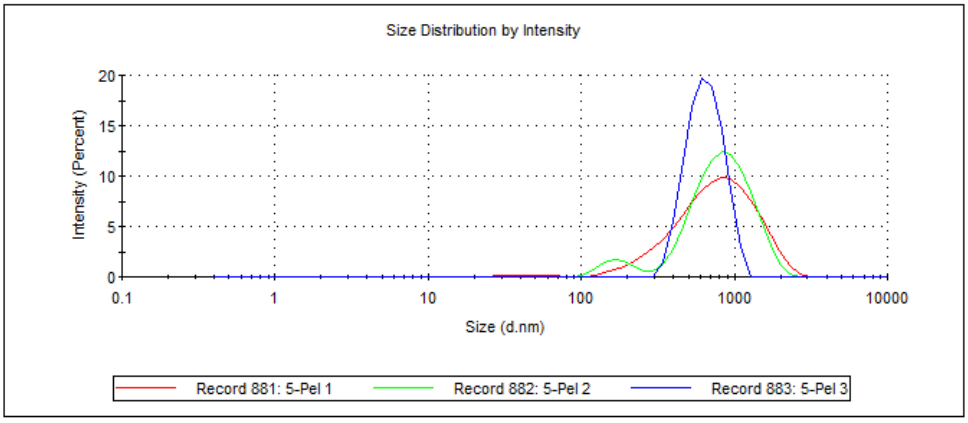


Figure 2: Particle Size and particle size distribution of CMPLGA(LT) pellet nanoparticles suspension

Sample Name:	4-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	869	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	18 November 2019 13:36

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	317.7	**Measurement Position (mm):**	1.05
Cell Description:	Disposable sizing cuvette	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	414.5	**Peak 1:**	517.2	100.0	202.8
PdI:	0.192	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.951	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

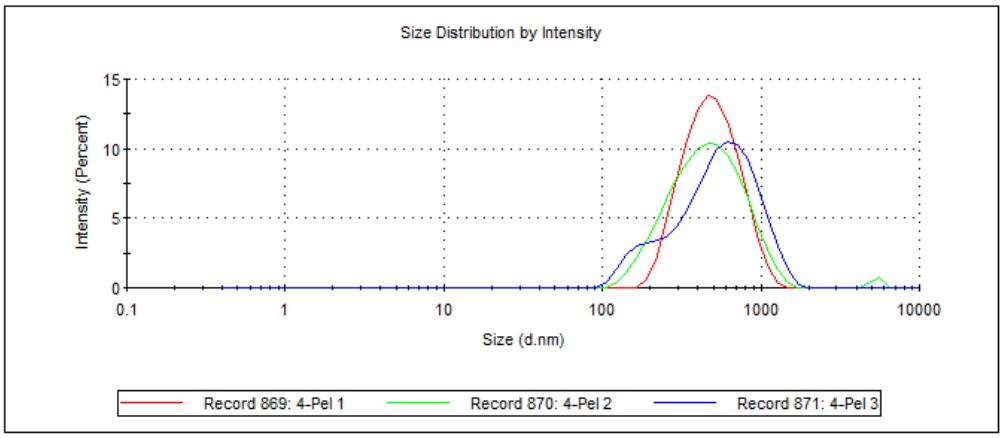


Figure 3: Particle size and particle size distribution of CMPLGA(LS) pellet nanoparticles

Sample Name:	4-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	872	**Dispersant RI:**	1.330
Date and Time:	18 November 2019 13:40	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	202.5	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	12.1	**Peak 1:**	12.1	100.0	4.87
Zeta Deviation (mV):	4.87	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0189	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

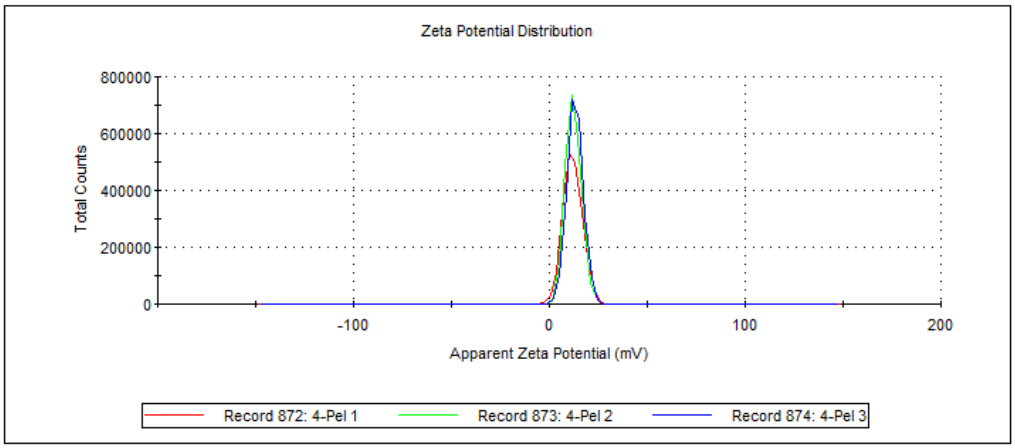


Figure 4: Particle zeta potential of CMPLGA(LS) pellets nanoparticle

Sample Name:	3-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	860	**Dispersant RI:**	1.330
Date and Time:	18 November 2019 13:12	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	162.5	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	-2.05	**Peak 1:**	-2.05	100.0	3.64
Zeta Deviation (mV):	3.64	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0107	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

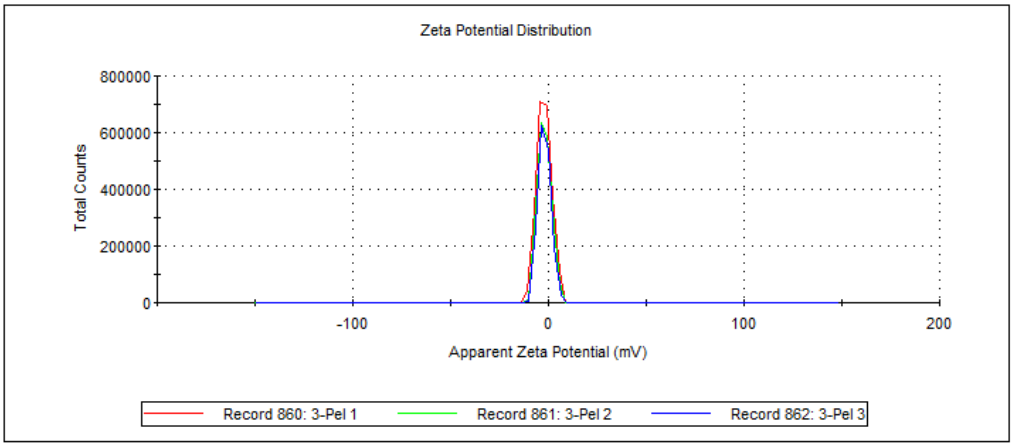


Figure 5: Particle zeta potential of CMPLGA(C) pellet nanoparticles

Sample Name:	3-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	857	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	18 November 2019 13:07

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	367.3	**Measurement Position (mm):**	1.05
Cell Description:	Disposable sizing cuvette	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	1505	**Peak 1:**	1665	100.0	301.8
PdI:	0.203	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.934	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

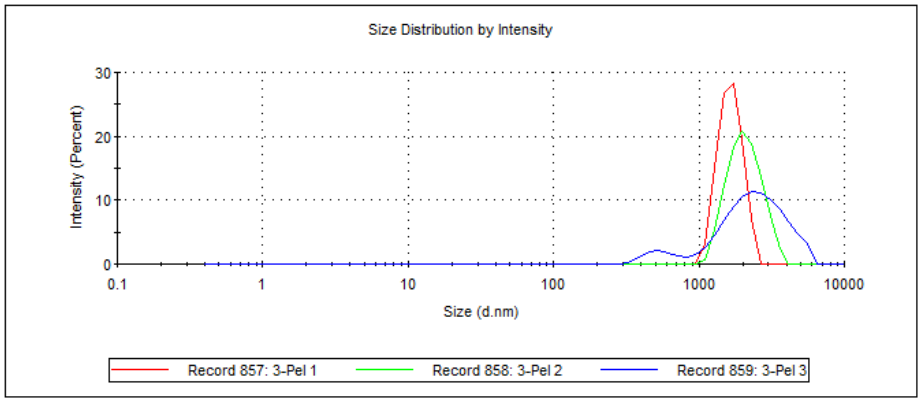


Figure 6: Particle size and particle size distribution of CMPLGA(C) pellet nanoparticles suspension

Sample Name:	2-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	848	**Dispersant RI:**	1.330
Date and Time:	18 November 2019 12:45	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	75.8	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	1.66	**Peak 1:**	1.66	100.0	3.31
Zeta Deviation (mV):	3.31	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0430	**Peak 3:**	0.00	0.0	0.00

Result quality : See result quality report

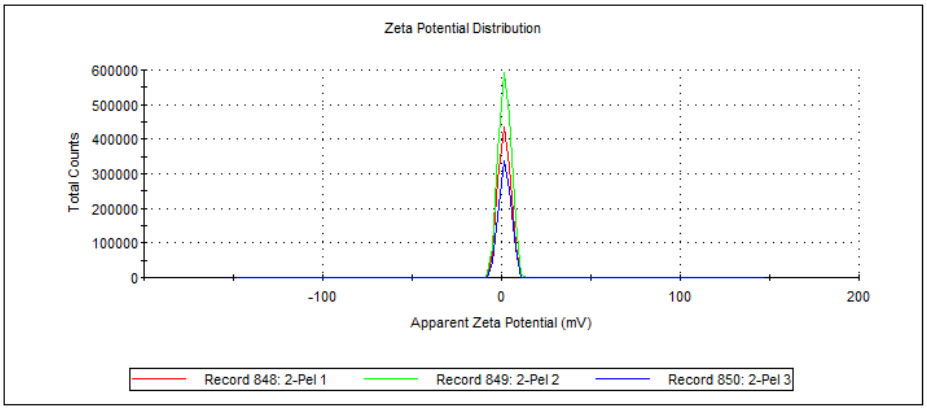


Figure 7: Particle zeta potential of CMPLGA(T) pellet nanoparticles suspension

Sample Name:	2-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	845	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	18 November 2019 12:36

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	112.2	**Measurement Position (mm):**	0.85
Cell Description:	Disposable sizing cuvette	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	1514	**Peak 1:**	2564	90.8	864.2
PdI:	0.503	**Peak 2:**	185.1	9.2	31.77
Intercept:	0.921	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

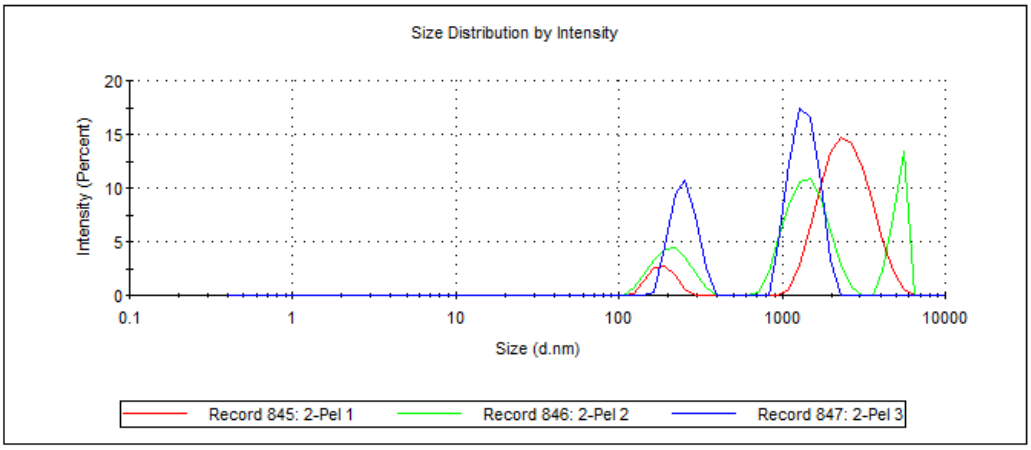


Figure 8: Particle size and particle size distribution of CMPLGA(T) pellet nanoparticles suspension

Sample Name:	1-Pel 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	836	**Dispersant RI:**	1.330
Date and Time:	18 November 2019 12:15	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	252.5	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	13.2	**Peak 1:**	13.2	100.0	3.89
Zeta Deviation (mV):	3.89	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0249	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

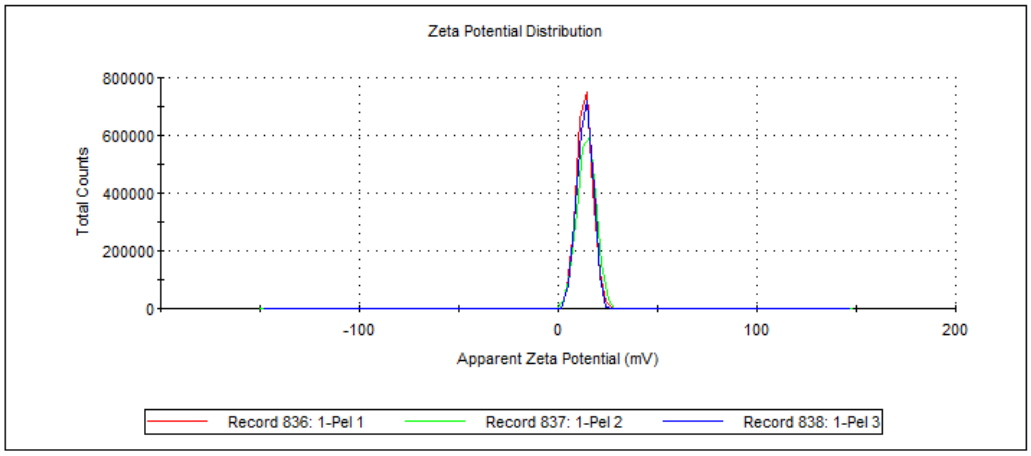


Figure 9: Particle zeta potential of CMPLGA(G) pellet nanoparticles suspension

C: After freeze drying (CHAPTER 6)

Sample Name:	BRL 56 initial suspension 3		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	1020	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 16:29

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	255.0	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	3162	**Peak 1:**	286.3	100.0	52.50
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.310	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

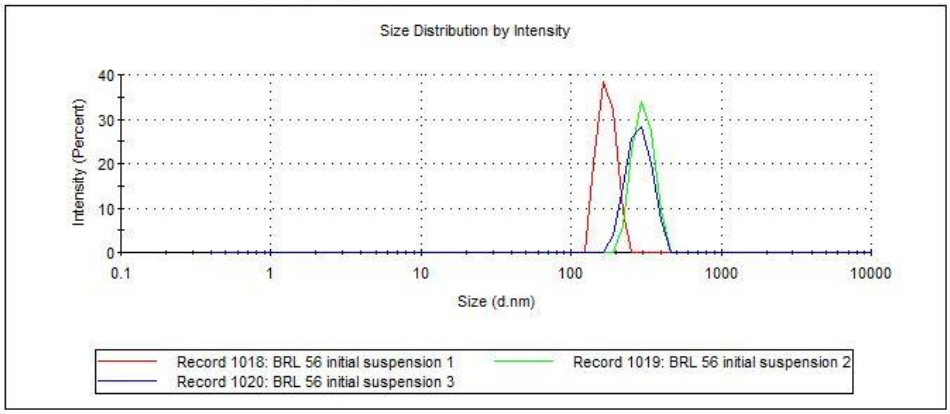


Figure 1: Particle size and particle size distribution of CMPLGA(C) initial nanoparticles suspension

Sample Name:	Tina-BRL56-initial sus 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	997	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 15:04	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	12
Count Rate (kcps):	366.5	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	10

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	-3.11	**Peak 1:**	-3.11	100.0	4.79
Zeta Deviation (mV):	4.79	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0198	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

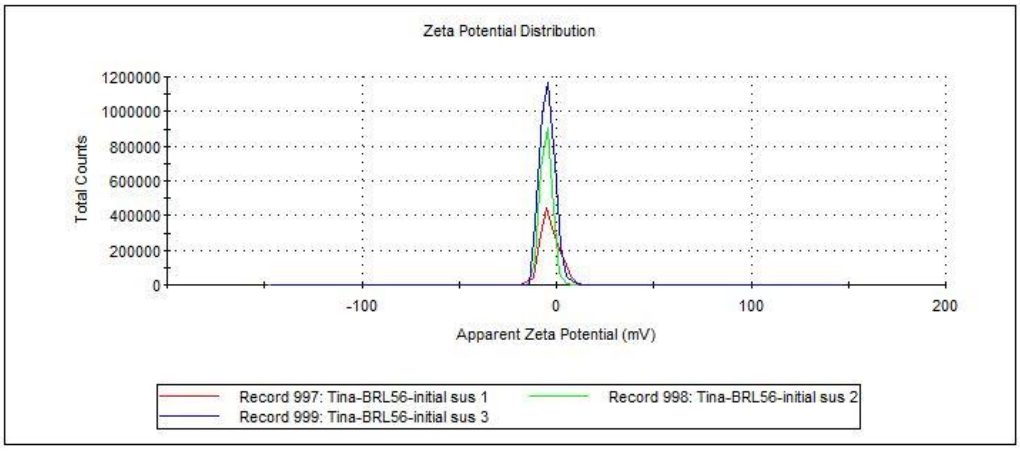


Figure 2: Particle zeta potential of CMPLGA(C) initial nanoparticles suspension

Sample Name:	Tina-BRL 40-SM-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	961	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 13:31

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	325.7	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	2362	**Peak 1:**	177.3	100.0	12.98
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.852	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

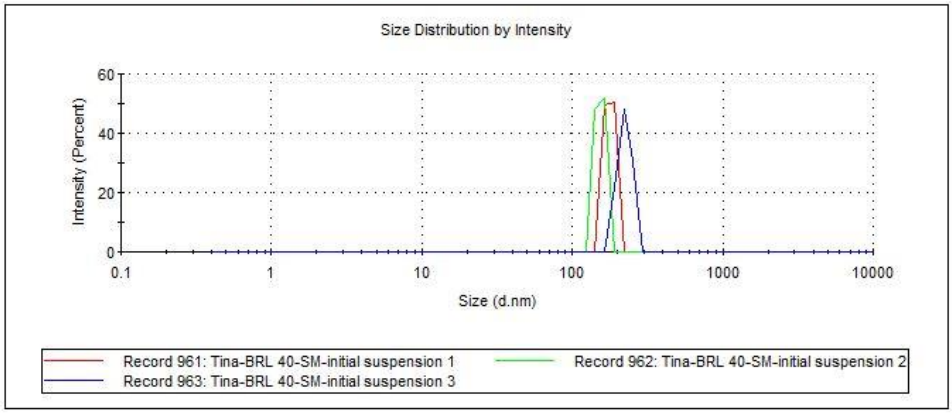


Figure 3: Particle size and particle size distribution of CMPLGA(T) initial nanoparticles suspension

Sample Name:	Tina-BRL 40-SM-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	964	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 13:40	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	15
Count Rate (kcps):	98.8	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	-13.2	**Peak 1:**	-13.2	100.0	3.77
Zeta Deviation (mV):	3.77	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0117	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

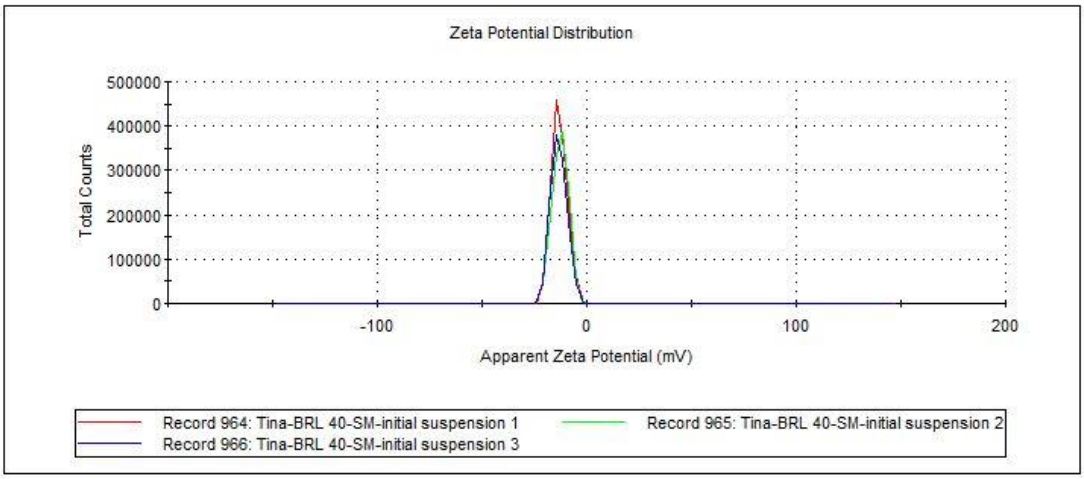


Figure 4: Particle zeta potential of CMPLGA(T) initial nanoparticles suspension

Sample Name:	Tina-BRL 40-SM-supernatant 1			
SOP Name:	mansettings.nano			
File Name:	Vusani.	**Dispersant Name:**	Water	
Record Number:	958	**Dispersant RI:**	1.330	
Material RI:	1.50	**Viscosity (cP):**	0.8872	
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 13:24	
Temperature (°C):	25.0	**Duration Used (s):**	50	
Count Rate (kcps):	256.4	**Measurement Position (mm):**	5.50	
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9	

		Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm): 321.5	**Peak 1:**	464.2	93.9	319.5
PdI: 0.453	**Peak 2:**	4666	6.1	800.4
Intercept: 0.839	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

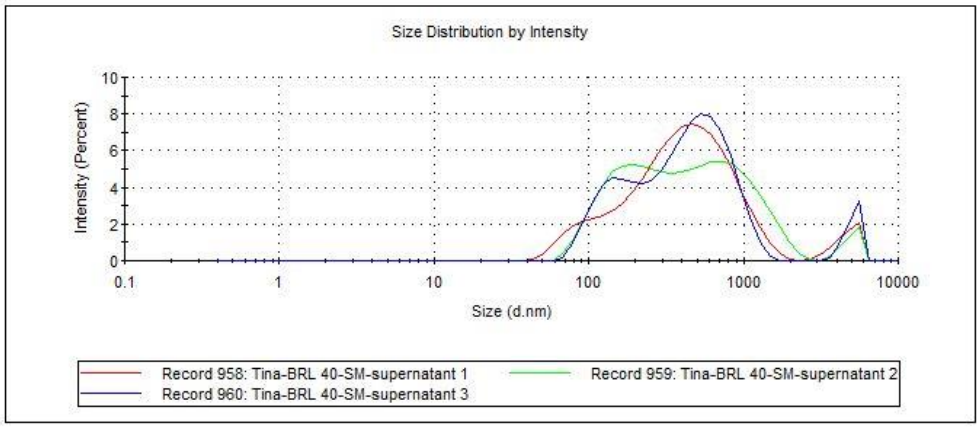


Figure 5: Particle size and particle size distribution of CMPLGA(T) supernatant nanoparticles suspension

Sample Name:	Tina-BRL 40-SM-supernatant 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	955	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 13:18	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	15
Count Rate (kcps):	129.9	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	11

		Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	-14.5	**Peak 1:** -14.5	100.0	5.42
Zeta Deviation (mV):	5.42	**Peak 2:** 0.00	0.0	0.00
Conductivity (mS/cm):	0.0158	**Peak 3:** 0.00	0.0	0.00

Result quality : Good

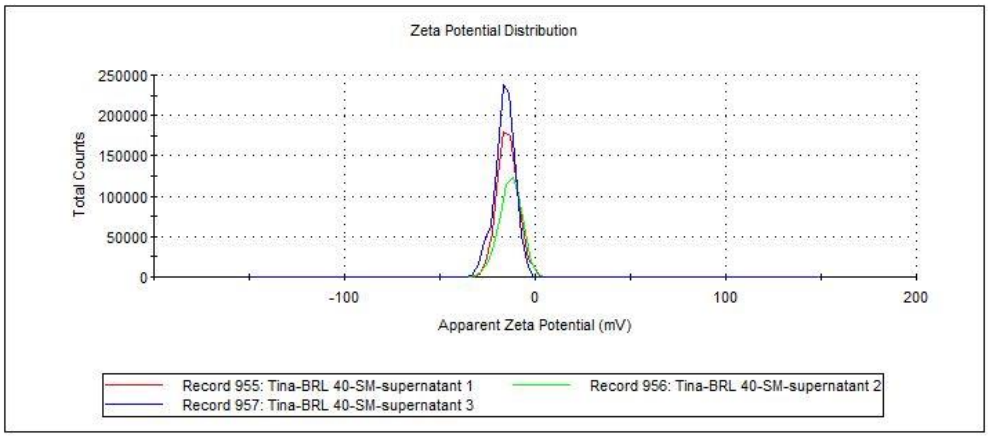


Figure 6: Particle zeta potential CMPLGA(T) supernatant nanoparticles suspension

Sample Name:	Tina-BRL33-Supernatant 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	979	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 14:15	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	101.3	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	10

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	-7.71	**Peak 1:**	-8.43	97.0	6.28
Zeta Deviation (mV):	8.31	**Peak 2:**	13.0	2.5	2.52
Conductivity (mS/cm):	0.00885	**Peak 3:**	55.4	0.5	0.00

Result quality : Good

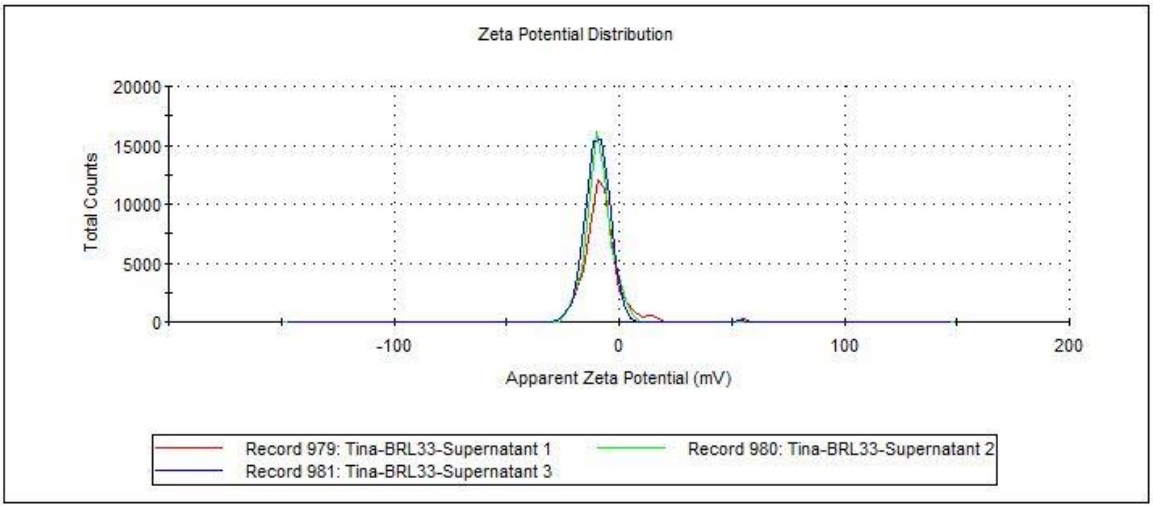


Figure 7: Particle zeta potential of CMPLGA(G) supernatant nanoparticles suspension

Sample Name:	Tina-BRL33-Initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	985	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 14:30

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	188.6	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	3301	**Peak 1:**	1201	100.0	165.2
PdI:	0.747	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.772	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

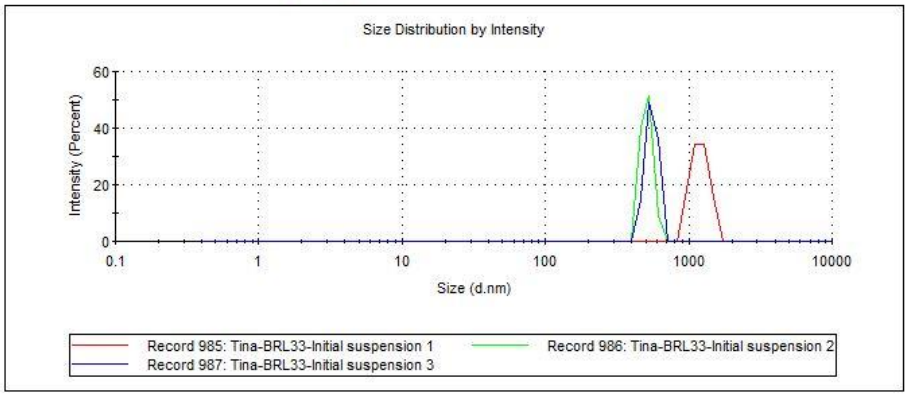


Figure 8: Particle size and particle size distribution of CMPLGA(G) initial nanoparticles suspension

Sample Name:	Tina-BRL33-Initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	982	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 14:20	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	190.2	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	5.38	**Peak 1:**	5.38	100.0	4.80
Zeta Deviation (mV):	4.80	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.00866	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

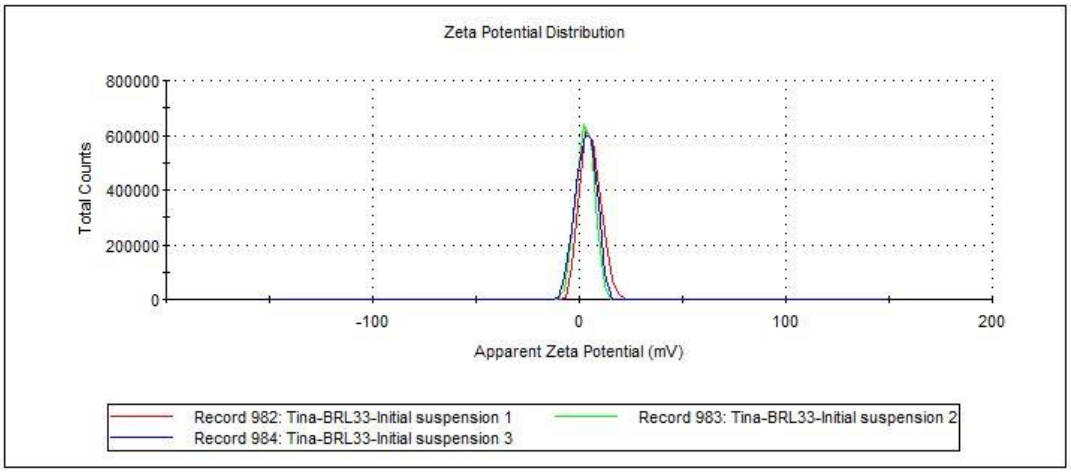


Figure 9: Particle zeta potential of CMPLGA(G) initial nanoparticles suspension

Sample Name:	EE supernatant of formulation 3		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	1014	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 16:11

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	415.4	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	233.6	**Peak 1:**	318.7	98.5	168.8
PdI:	0.387	**Peak 2:**	5237	1.5	452.2
Intercept:	0.874	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

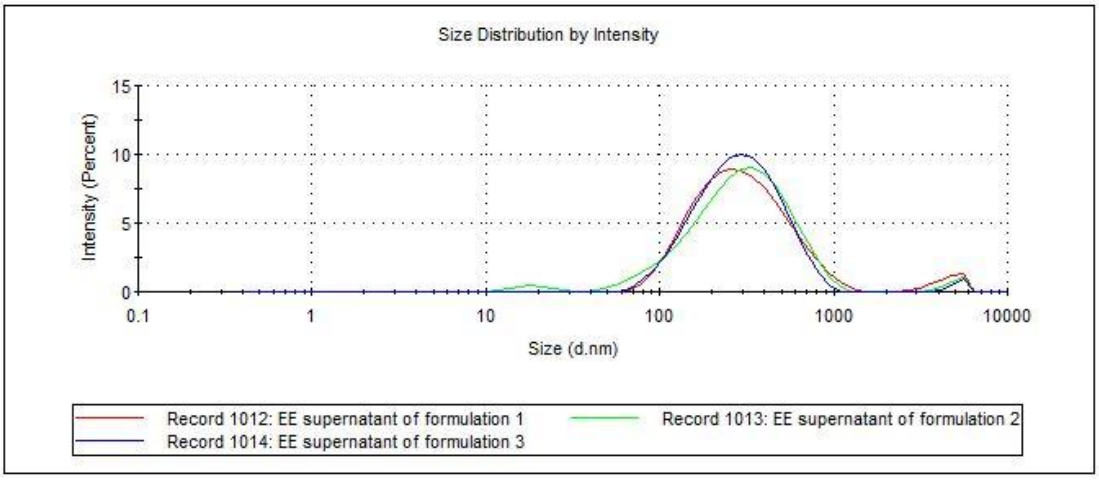


Figure 10: Particle size and particle size distribution of CMPLGA(EE) initial formulation nanoparticles suspension

Sample Name:	EE pellet 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	1003	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 15:41

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	440.7	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	8

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	574.6	**Peak 1:**	573.1	90.1	151.6
PdI:	0.439	**Peak 2:**	117.2	9.9	20.82
Intercept:	0.873	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

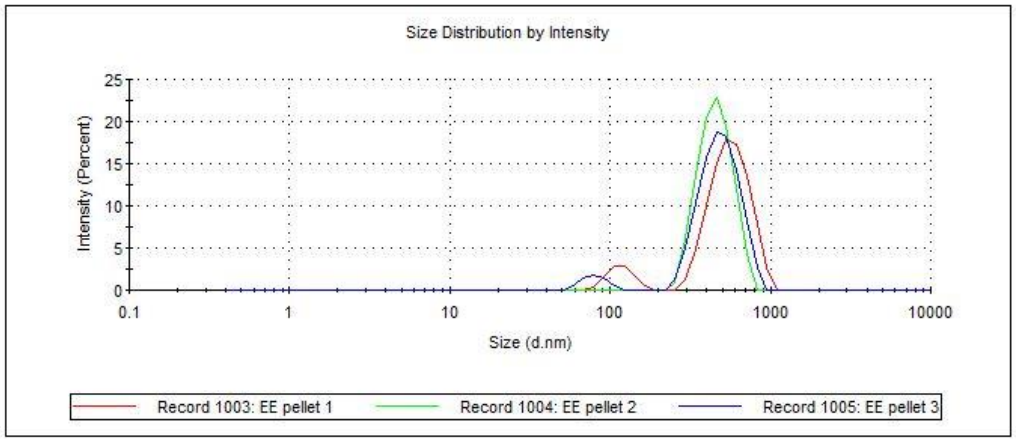


Figure 11: Particle size and particle size distribution of CMPLGA(EE) pellet nanoparticles suspension

Sample Name:	EE pellet 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	1006	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 15:47	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	12
Count Rate (kcps):	118.3	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9

		Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	36.6	**Peak 1:**	36.6	100.0	5.36
Zeta Deviation (mV):	5.36	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0363	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

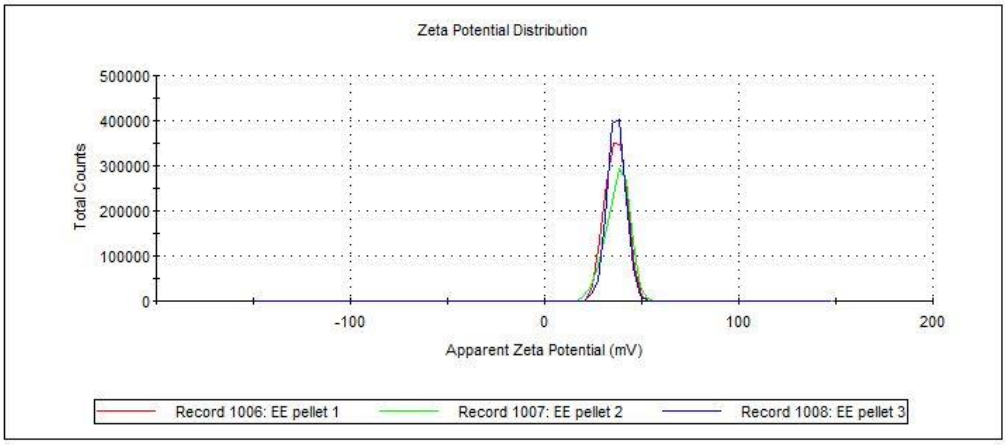


Figure 12: Particle zeta potential of CMPLGA(EE) initial nanoparticles suspension

Sample Name:	Tina-EE-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	973	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 13:57

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	176.9	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	2807	**Peak 1:**	355.3	100.0	29.64
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.875	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

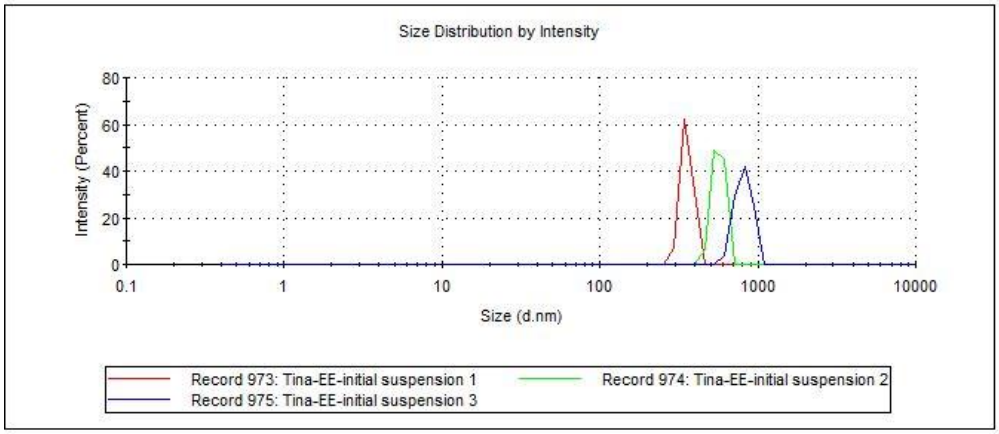


Figure 13: Particle size and particle size distribution of CMPLGA(EE) initial nanoparticles suspension

Sample Name:	Tina-EE-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	976	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 14:03	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	163.6	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9

			Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	6.36	**Peak 1:**	6.36	100.0	4.74
Zeta Deviation (mV):	4.74	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0269	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

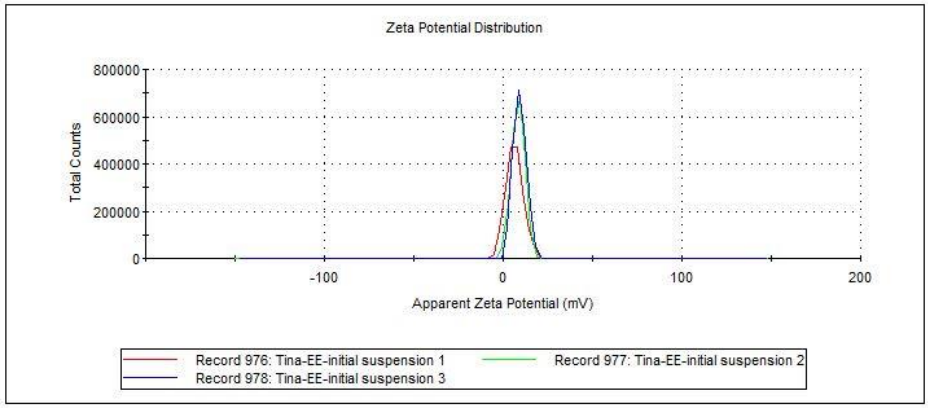


Figure 14: Particle zeta potential of CMPLGA(EE) initial nanoparticles suspension

Sample Name:	Tina-BRL 59-TM-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	949	**Dispersant RI:**	1.330
Material RI:	1.50	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.100	**Measurement Date and Time:**	28 November 2019 13:07

Temperature (°C):	25.0	**Duration Used (s):**	50
Count Rate (kcps):	228.2	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	4301	**Peak 1:**	2049	100.0	222.8
PdI:	0.231	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.894	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report

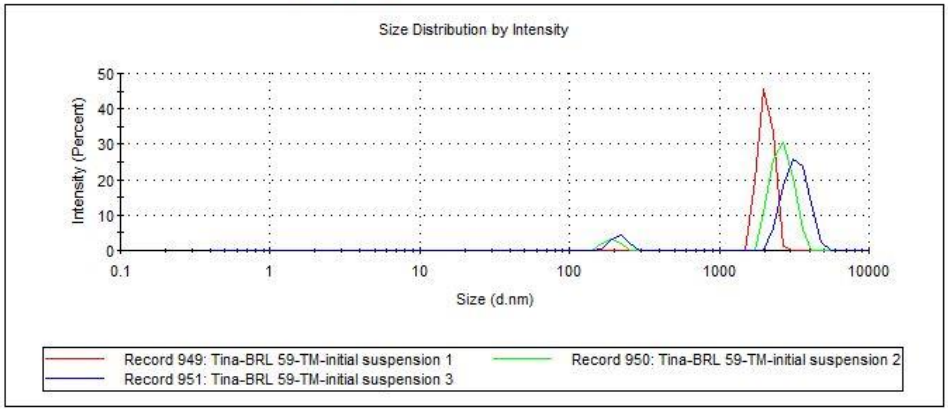


Figure 15: Particle size and particle size distribution of CMPLGA(LT) initial nanoparticles suspension

Sample Name:	Tina-BRL 59-TM-initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	952	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 13:14	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	15
Count Rate (kcps):	212.6	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	40.2	**Peak 1:**	40.2	100.0	3.27
Zeta Deviation (mV):	3.27	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.00879	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

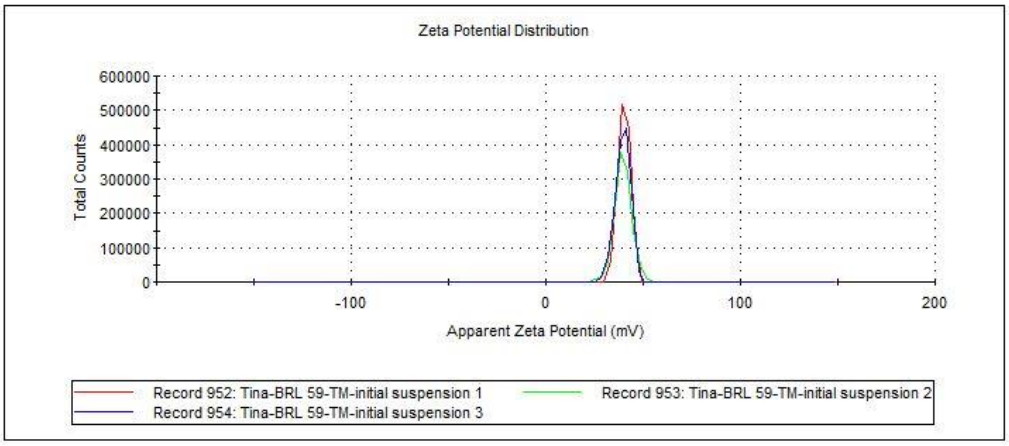


Figure 16: Particle zeta potential of CMPLGA(LT) initial nanoparticles suspension

Sample Name:	Tina-BRL 59-SM-Initial suspension 1		
SOP Name:	mansettings.nano		
File Name:	Vusani.	**Dispersant Name:**	Water
Record Number:	940	**Dispersant RI:**	1.330
Date and Time:	28 November 2019 12:34	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

Temperature (°C):	25.0	**Zeta Runs:**	15
Count Rate (kcps):	115.9	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

		Mean (mV)	**Area (%)**	**St Dev (mV)**
Zeta Potential (mV):	6.86	**Peak 1:** 6.86	100.0	3.51
Zeta Deviation (mV):	3.51	**Peak 2:** 0.00	0.0	0.00
Conductivity (mS/cm):	0.00507	**Peak 3:** 0.00	0.0	0.00

Result quality : Good

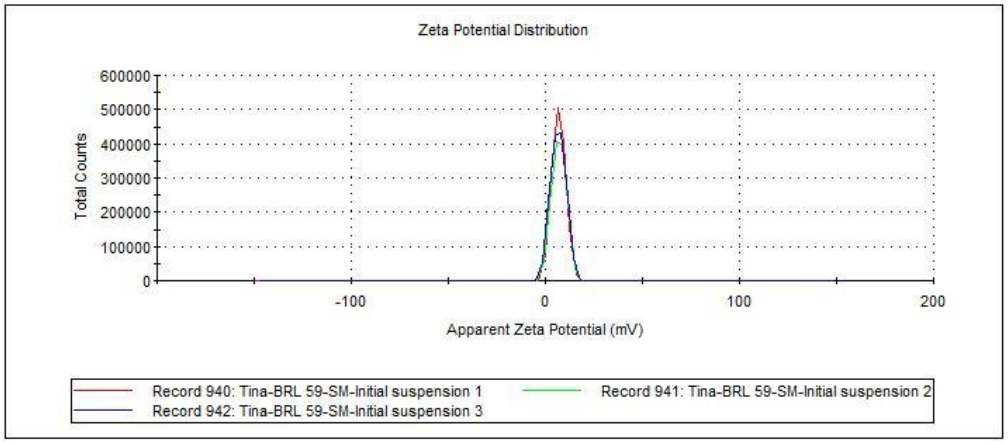


Figure 17: Particle zeta potential of CMPLGA(LS) initial nanoparticles suspension

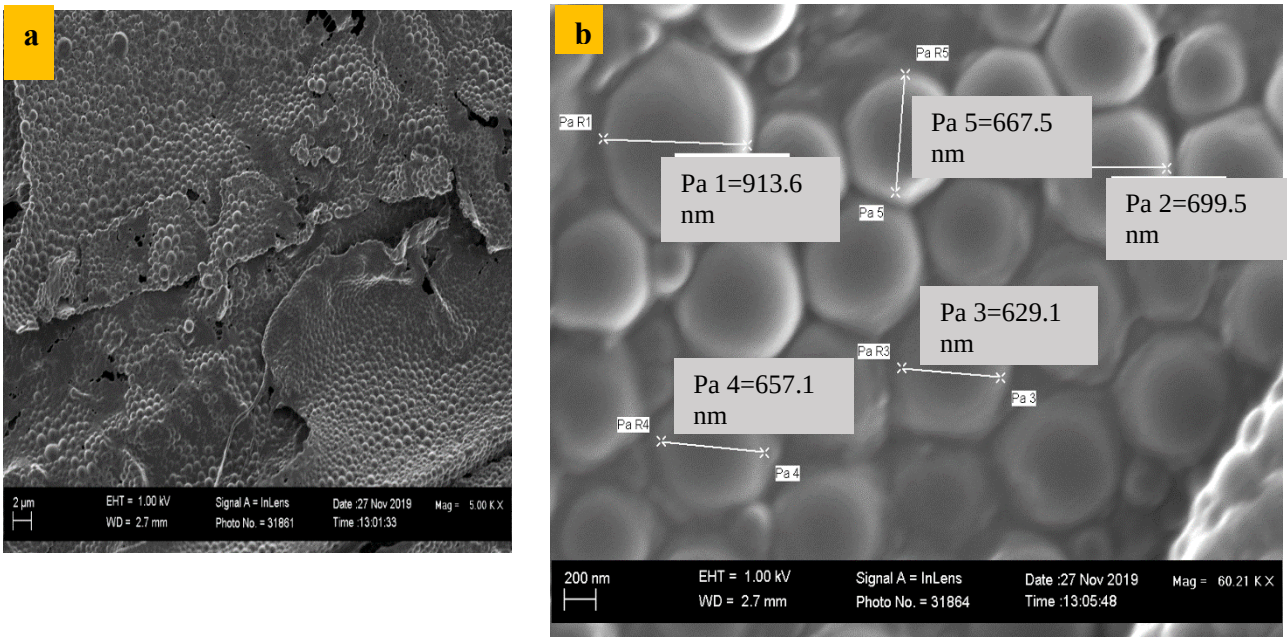


Figure 18: Scanning Electron Microscopy (SEM) image of CMPLGA(C) (a) Particle morphology at 5.0 KX magnification, (b) Particle morphology at 60.21 KX magnification (Pa= Particle diameter and Pb= particle degree.

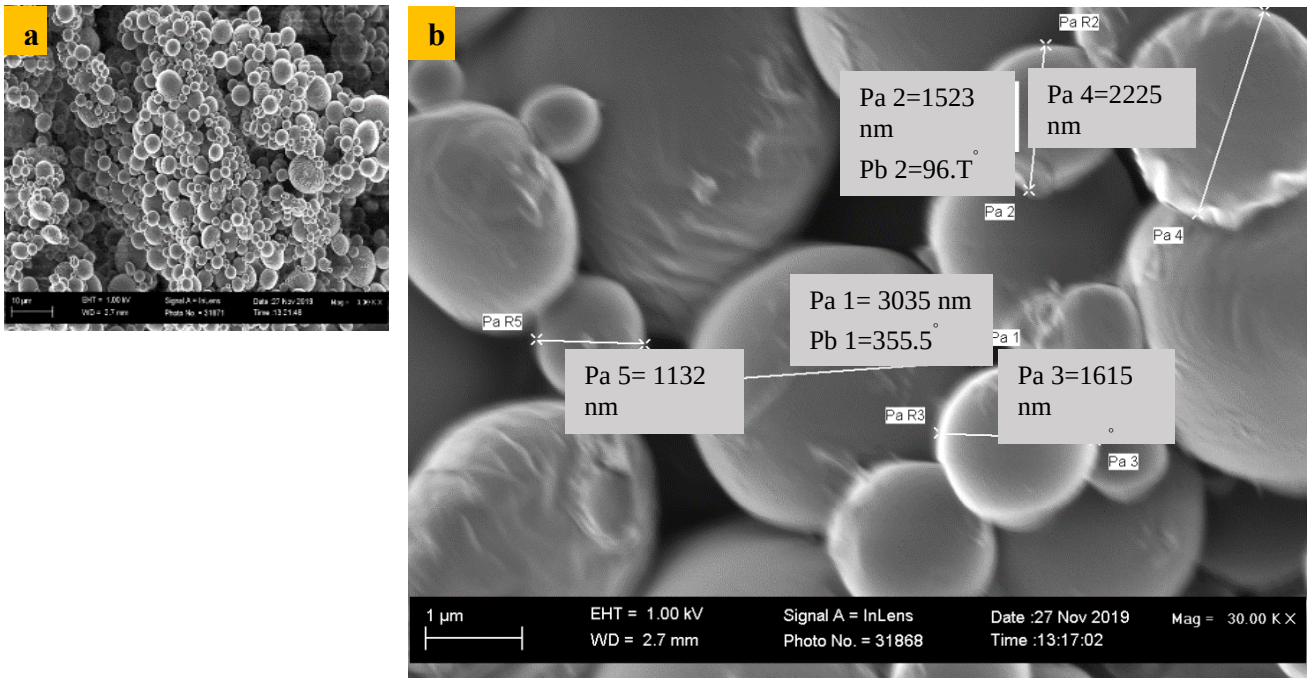


Figure 19: Scanning Electron Microscopy (SEM) image of CMPLGA(LT) (a) Particle morphology at 5.0 KX magnification, (b) Particle morphology at 60.21 KX magnification (Pa= Particle diameter and Pb= particle degree.

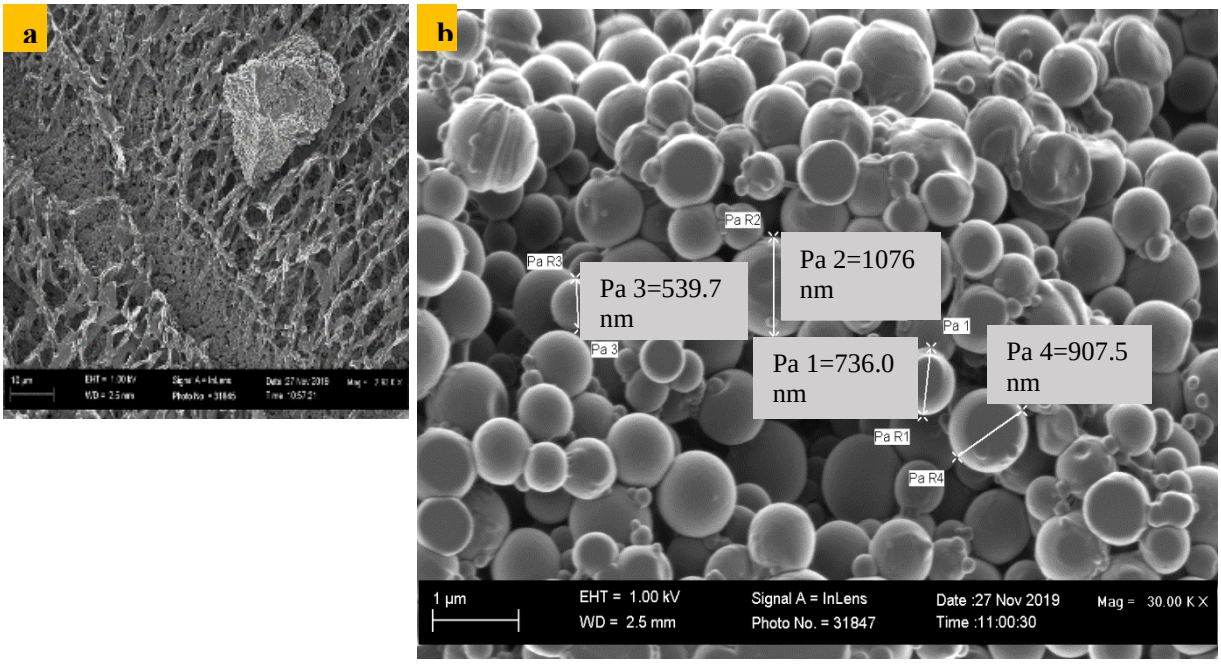


Figure 20: Scanning Electron Microscopy (SEM) image of CMPLGA(LS) (a) Particle morphology at 5.0 KX magnification, (b) Particle morphology at 60.21 KX magnification (Pa= Particle diameter and Pb= particle degree.

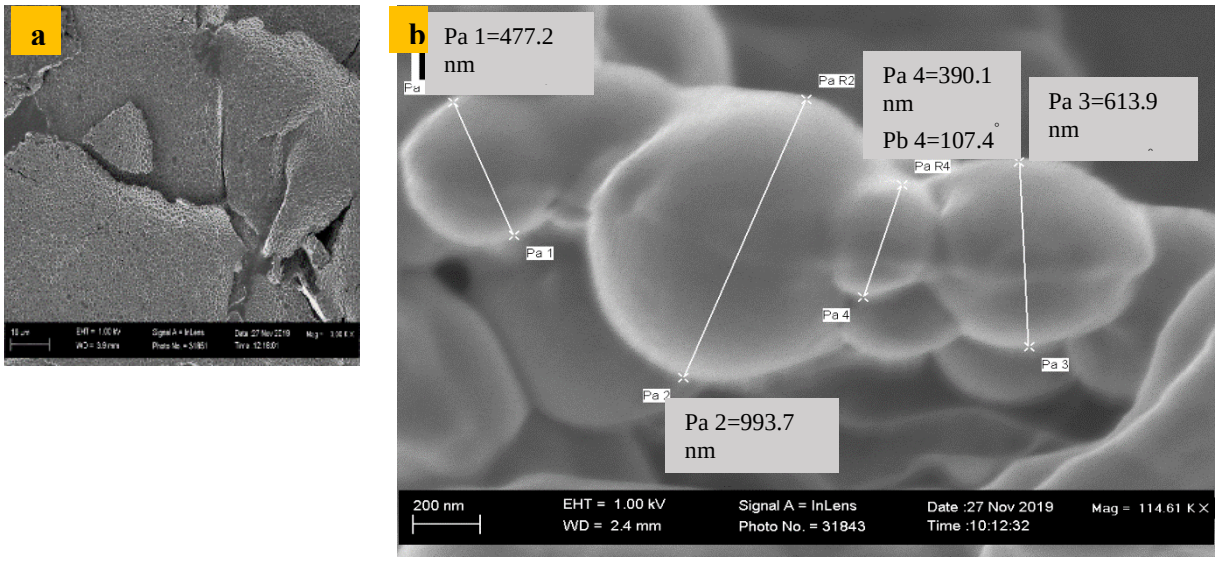


Figure 21: Scanning Electron Microscopy (SEM) image of CMPLGA(G) (a) Particle morphology at 5.0 KX magnification, (b) Particle morphology at 60.21 KX magnification (Pa= Particle diameter and Pb= particle degree.

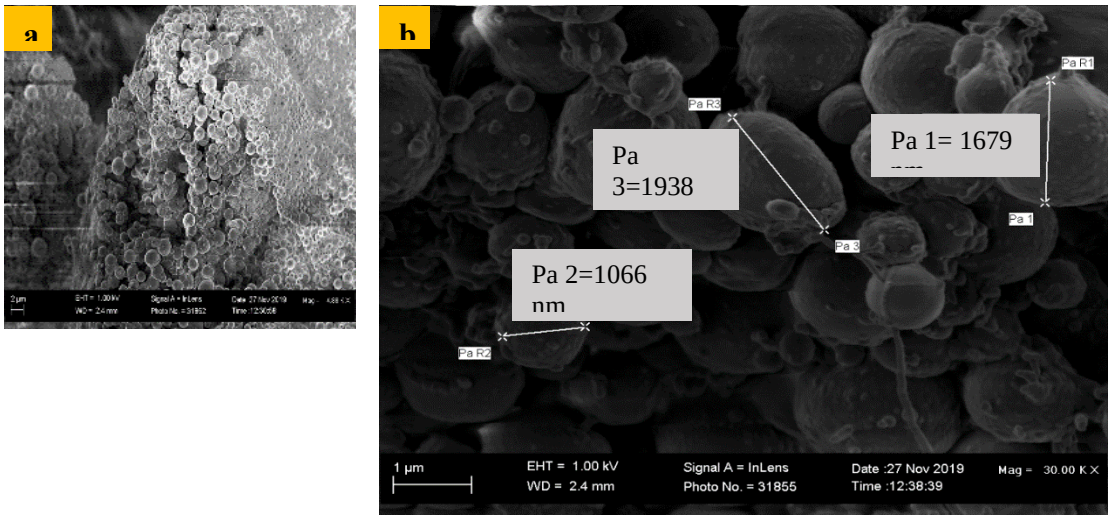


Figure 22: Scanning Electron Microscopy (SEM) image of CMPLGA(EE) (a) Particle morphology at 5.0 KX magnification, (b) Particle morphology at 60.21 KX magnification (Pa= Particle diameter and Pb= particle degree.

Appendix K: Resazurin-based MIC test *Salmonella* and *E. coli* CHAPTER 6

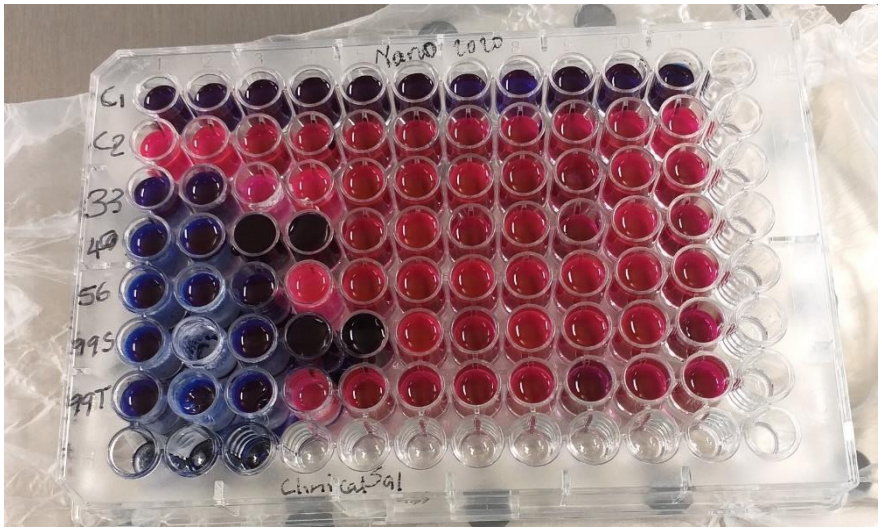


Figure 1: MIC test of Nanoparticles against MDR clinical *E. coli* by resazurin-based assay on a 96 well plate

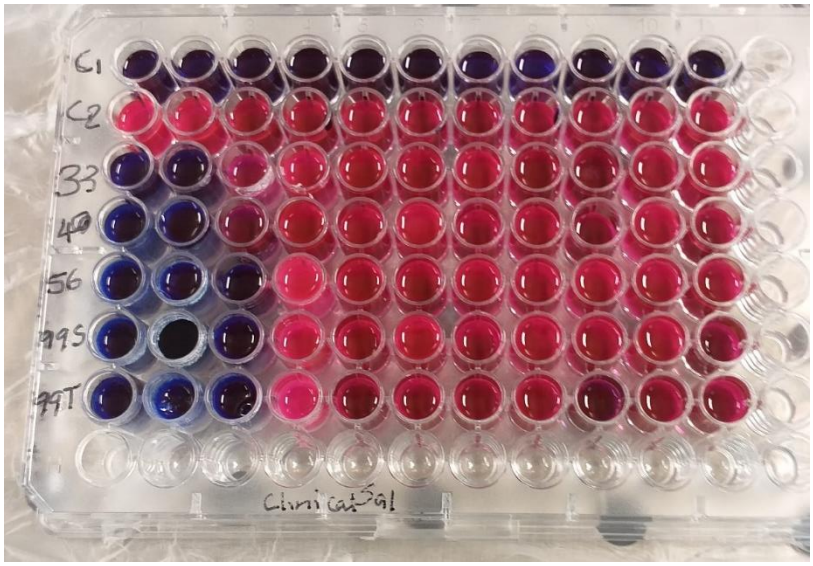


Figure 2: MIC test of Nanoparticles against MDR Clinical *Salmonella* isolates by resazurin-based assay on a 96-well plate.