EFFECT OF INCUBATION PERIOD ON THE ANTIBACTERIAL ACTIVITY OF MAGNESIUM CHLORIDE NANOPARTICLES ON BACTERIA ISOLATED FROM A DISEASED *Manihot esculenta*

BY

Chiamaka Juliet EDOZIEM (MISS)

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CERTIFICATION

This is to certify that this project was carried out by Chiamaka Juliet EDOZIEM in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin city under my supervision.

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Mr. G.O. ORIBHABOR **DATE**

APPROVAL

This project work is accepted in partial fulfillment for the award of Barchelor of Science, Bsc(Hons) in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City

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PROF.(Mrs). F.I. AKINNIBOSUN **DATE**
(Head of Department)

DEDICATION

This work is dedicated to God Almighty for his grace and wisdom during the course of this project and to my parents and friends for their unending support all through this journey.

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ABSTRACT

Nanotechnology is an emerging field of science that includes synthesis and development of various nano-materials. The aim of this study was to determine the effects of incubation period on the antimicrobial activities of magnesium chloride nanoparticles on bacteria isolated from a diseased *Manihot esculenta* leaf. Three bacteria were isolated from the diseased *Manihot esculenta* leaf obtained from three different farms. The antibacterial activities of *Moringa oleifera* and aqueous extract were performed using agar well diffusion method. From the results, no activity was recorded against the test isolates (0.00+0.00 cm) but this was significantly different from the control which showed zone of inhibition ranging from 2.60+ 0.10 cm for *Enterobacter Ludwigii GM7* to 3.20+0.10cm for *Erwinia spp* after incubation periods of 24hrs and 48hrs respectively. The result of this study reveals that magnesium chloride nanoparticles (at all concentrations) had no effect on the pathogens. Further studies should be however conducted to unravel the reasons behind this resistance by the test isolates against synthesized MgONp.

CHAPTER ONE

INTRODUCTION

Nanotechnology is an emerging field of science that includes synthesis and development of various nanomaterials. These are small structures and small-sized materials of dimensions in the range of few nanometers to less than 100 nanometers. Nanoparticles (NPs) show unique and considerably changed chemical, physical, and biological properties compared to bulk of the same chemical composition, due to their high surface-to-volume ratio. It is considered as a field of science widely subjugated in many scientific fields. NPs exhibit size and shape-dependent properties which are of interest for applications ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes. There is a strong demand to develop novel antimicrobial materials, and the emergence of nanotechnology is creating a variety of options in this respect. Promising approaches for the effective delivery of therapeutic compounds can be provided by the use of nanoparticles as drug carriers. These particles also have many applications in different fields such as medical imaging, nanocomposites, filters, drug delivery, and hyperthermia of tumors (Salata, 2004).

The existing disadvantages of conventional antibiotics can be solved to some extent by using nanomaterial-based antimicrobial delivery systems. There has been a plethora of reports of several clinical advantages of antimicrobial nanoparticles and their utilization as drug carrier systems. Numerous nanoparticles exhibit antibacterial activity against several bacterial species. Today, nanomaterials are a promising platform to control bacterial infections in a broad range of applications, and particularly for agricultural purposes (Maria *et al*., 2019)

Some views have classified nanoparticles based on how they are formed; as natural, incidental, and engineered. Naturally occurring nanoparticles are shaped due to natural process without any human intervention. Engineered nanoparticles comprise of any manufactured particles with nanoscale dimensions that are produced intentionally for commercial or research application (Nadim *et al*.,2008)

In order to solve the problem of bacterial pathogen of plants, it is highly necessary to develop effective antimicrobial agents to control the bacterial population (Kumar *et al*., 2008). Nanoparticles are recognized as antibacterial agents due to their size, structure, and surface properties. Antimicrobial nanoparticles are of great interest as they provide a number of benefits over free antimicrobial agents. Thus, nanotechnology offers a way to improve the activity of inorganic antibacterial agents. Administration of antimicrobial agents using nanoparticles can increase the overall pharmacokinetics by progressing therapeutic index, extending drug circulation, and providing controlled drug release. In such approaches, the conventional antibiotics can be loaded into the nanoparticles through physical encapsulation, adsorption, or chemical conjugation (Kumar *et al*.,2008)

Magnesium chloride is the name for the chemical compound with the formula $MgCl_2$ and its various hydrates $MgCl_2(H_2O)x$. Magnesium is ordinarily a reported element essential for plant growth and flourishing, especially for its foliar growth and photosynthesis. $MgCl_2$ solution has been found to be most effective as it had two main advantages—it was not harmful for the tissue and it highly increased leucocyte activity and phagocytosis. Magnesium salts are typically associated with positive effects on microbial cells. The development of nanoscale particles of Mg, Fe or their oxides may help in triggering the metabolic pathways leading to better growth and higher yields of plants

In detail, nanocarriers can conquer the solubility and stability issues and reduce side effects associated with conventional antimicrobials (Lemire *et al*., 2013). With the use of nanocarriers in the delivery of drugs, combination drug therapy can be achieved by incorporating two or more drugs or different therapeutic modalities into the carrier matrix. (Lemire *et al*.,2013)

There is an increasing excitement in the study of nano-scale matter for the enhancement of plant productivity, and treatment of several diseases of plants. This increasing interest prompted this study which seeks to take advantage of changing incubation period to ascertain better antimicrobial activity of magnesium chloride nanoparticles against selected plant pathogenic bacteria. (Demishtein *et al*,2019)

1.1 Aim and Objectives of Study

The aim of this study was to determine the effects of incubation period on the antimicrobial activity of magnesium chloride nanoparticles on bacteria isolated on a diseased *Manihot esculenta* leaf

The objectives of this study were to:

i. isolate pathogen from the diseased *Manihot esculenta* leaf
ii. biosynthesize magnesium chloride nanoparticles using magnesium chloride aqueous solution
iii. characterize magnesium chloride nanoparticles using UV-Vis spectrophotometer
iv. determine the antibacterial activities of magnesium chloride nanoparticles against test isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Nanotechnology and Nanoparticles

Nanoscience and nanotechnology has attracted a great interest over the last few years due to its potential impact on many scientific areas such as energy, medicine, pharmaceutical industries, electronics, and space industries (Iravani, 2014). Nanotechnology is an emerging field of science that includes synthesis and development of various nanomaterials. Nanotechnology is manipulation of matter on an atomic, molecular and supramolecular scale (Mousavi *et al*., 2011). Nanotechnology is developing as the sixth revolutionary technology in the current era. It is considered as a field of science widely subjugated in many scientific fields (Mousavi *et al*., 2011). This technology deals with small structures and small-sized materials of dimensions in the range of few nanometers to less than 100 nanometers (Iravani, 2014). At the nanoscale, the matter presents altered properties which are novel and very much different from those observed at the macroscopic level (Guiterrez *et al*., 2011). Nanoparticles (NPs) show unique and considerably changed chemical, physical, and biological properties compared to bulk of the same chemical composition, due to their high surface-to-volume ratio. These particles also have many applications in different fields such as medical imaging, nanocomposites, filters, drug delivery, and hyperthermia of tumors (Salata, 2004). The physical properties of nanoparticles are different from the properties of the bulk material. According to (2014), NPs exhibit size and shape-dependent properties which are of interest for applications ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes.

2.2 Background on the uses of Nanoparticles

Nanotechnology is a quickly progressing area with various uses in the biomedical sciences and involves engineering and production of materials at the atomic and molecular level. An important area of research in nanoscience deals with the synthesis of nanometer-size particles of different morphologies, sizes, and monodispersity (Sastry, 2003). This enables a broad range of possible applications, including cosmetic, pharmaceutical, and medical utilization (Semenzin *et al*., 2015). In this regard, there is a growing need to develop reliable, nontoxic, clean, ecofriendly, and green experimental protocols for the synthesis of NPs. One of the options to achieve this objective is to use natural processes such as use of enzymes, microbial enzymes, vitamins, polysaccharides, biodegradable polymers, microorganisms, and biological systems for synthesis of NPs.

According to Kaur *et al*. (2021), metal nanoparticles exhibit their applicability in various fields like chemistry, -optoelectronic, sensing device, image recording media, engineering science, medical field, electronics, medicine and in pharmacological devolvement due to their specific properties such as higher surface area, antibacterial, anticancer, antimicrobial and antifungal acuities of the various metal nanoparticles like palladium-nanoparticles, platinum nanoparticles, gold-nanoparticles, etc, silver-nanoparticles have attracted special attention due to their excellent physicochemical properties, which include catalytic properties, electrical conductivity, thermal properties, surface Plasmon phenomenon and biological properties. The extensive interest of Nps can be attributed to their tailorable physicochemical properties and wide range of applications in the pharmaceutical and industrial fields.

The revolution of metal-based nanoparticles represents an important breakthrough in nanotechnology towards the production of high-quality products. A scientific survey indicates

that biowaste from fruits and vegetables peel rich in vast array of biomolecules are dumped in huge quantities without management which ultimately leads to the generation of unhygienic condition for the market vendors. The sustainable solutions for waste reduction can be established through its utilization for the production of valuable nanoparticles.

By twenty-first century, it is described as the wonder of medicine that leads to industrial revolution (Selim *et al*., 2020). Among the different approaches in skin rejuvenation, the use of nanoparticles (Nps) loaded with cosmeceuticals (e.g., phytochemicals, vitamins, and hyaluronic acid) has become an interesting alternative. Importanly, plant based Nps are a valuable source of myriad bioactive metablolites as ascorbic acid, flavonoids, alkaloids and terpenoids. They serve as a natural bio-reductaned of metal ions as well as capping agents for sterically stabilizing Nps through the reduction of the direct interaction between molecules (Jain *et al*., 2021).

Metal nanoparticles synthesis using biological agents which are less harmful and non-toxic, termed as green chemistry, involve agents like microorganisms, fungus, plant, and their products including the leaf extracts (Pandit, 2015). Recent studies report the synthesis of silver nanoparticles synthesis using leaf extracts from plants such as *Sorbus aucuparia* leaf, Aloe vera, Neem, ocimum leaf, kigelia Africana leaf extract and olive leaf, *Zingiberaceae* species, *Melia azedarach* (Kaur *et al*., 2021).

The benefits of nanoparticles (nanonutrients) over conventional fertilizers may be twofold i) due to small size they may have better permeability into a plant system and can be effective in extremely low doses and ii) availability of high surface area may provide more reaction sites resulting in increased photosynthetic efficiency of plants, leading to higher productivity per unit of land and energy (Kaul *et al*., 2012). Besides, their application may also help in minimizing the deleterious effects of conventional fertilizers (often applied in high doses) on biotic and abiotic

environment over a period of time. In the pharmaceutical field, silver nanoparticles have been intensively studied due to their unique physicochemical properties that utilized in many cosmetics preparations with a broad range of antibacterial activity (Younis *et al*., 2021).

2.3 Classification of Nanoparticles

Chapman *et al*. (2012) divided nanoparticles based on the material such as carbon-based, metal-based, composites, and dendrimers. Carbon-based nanoparticles may be hollow spheres, ellipsoid, or tube. Examples of this type of nanoparticles are spherical fullerenes and carbon nanotubes. Metals like gold (Au), silver (Ag) as well as reactive metal oxides like TiO_2 and ZnO nanoparticles fall into metal-based NPs. Composites are NPs combined with other nano-size atoms or with bulky atoms (e.g. spherical SiO_2 nanoparticles and polycaprolactone) and are used in medical devices as well as for packaging (lorens *et al*., 2012). The last classification is dendrimers and these are composed from nano-sized polymers and usually used as catalysts. Dendrimers are compatible with organic structure such as DNA and used particularly in medical and biomedical field e.g. drug delivery (Alkilany *et al*., 2012).

Some other views divided nanoparticles based on how they are formed; natural, incidental, and engineered. Naturally occurring nanoparticles are shaped due to natural process without any human intervention. Examples of naturally occurring nanoparticles include fires, viruses and volcanic ash (Chapman *et al*., 2012).

Engineered nanoparticles comprise of any manufactured particles with nanoscale dimensions that are produced intentionally for commercial or research application. Examples include controlled shape and size metals, semiconductors, electronics, and optical displays. Furthermore, nanoparticles separated based on their chemical composition into organic and inorganic (Nowack and Bucheli, 2007).

2.4. Sources of nanomaterials

2.4.1 Engineered Nanoparticles

According to Thalmann *et al*. (2016), simple combustion during cooking, in vehicles, fuel oil and coal for power generation. airplane engines, chemical manufacturing, welding, ore refining and smelting are some of the anthropogenic activities that lead to NP formation. NMs such as carbon NPs, TiO_2 NPs and hydroxyapatites are present in commercial cosmetics, sporting goods, sunscreen and toothpaste. Thus, these synthetic NPs are a new genre of NPs that may induce adverse environmental and human health effects.

2.4.2 Accidents and natural discharges

Photochemical reactions, volcanic eruptions, and forest fires are some of the natural processes that lead to the production of natural NPs as mentioned. Plants and animals, which is frequent in nature, contributes to NP composition in nature. Dust storms, volcanic eruptions, and forest fires are events of natural origin that are reported to produce high quantities of nanoparticulate matter that significantly affect worldwide air quality. Similarly, transportation, industrial operations, and charcoal burning are some of the human activities that lead to the emergence of synthetic NPs. Only about 10% of overall aerosols in the atmosphere are generated by human activity, whereas the naturally generated ones amount to 90% of atmospheric aerosols (Weil *et al*., 2015) .

Thus, pollution from vehicles is a major cause of nanoparticulate contamination in urban atmosphere (Metreveli *et al*., 2015). In cosmopolitan cities and town, the main source of atmospheric micro- and nanoparticles is automobile exhaust. Amongst the types of automobile exhaust, diesel engines release 20–130 nm sized particles whereas gasoline engines release 20–60 nm sized particles. According to Cornelis *et al*. (2013), more than 90% of carbon NPs present in the atmosphere are diesel-generated particles.

2.5 Synthesis of Nanoparticles

Currently, nanoparticles are produced by chemical or physical approaches which may leave increasing amount of toxic wastes and therefore, there is a growing need to develop environmentally benign nanoparticle synthesis processes that do not use toxic chemicals in the synthesis protocol (Sastry *et al*., 2003).

According to Kaur *et al.* (2021), recently, there have been considerable efforts to produce non-toxic, reliable, and eco-friendly methods for synthesis of nanoparticles with desired size and morphologies. Initiatives have been taken to use environment friendly methods in the research field to protect our global environment.

According to Kaur *et al.* (2012), both unicellular and multicellular organisms are reported in literature to produce inorganic materials either intra- or extra-cellularly. Some well-known examples of microbial synthesis of inorganic materials include magnetotactic bacteria which synthesize magnetite nanoparticles and S-layer bacteria which produce gypsum and calcium carbonate layers. Therefore, microbial systems are now being increasingly explored as safer alternative for production of nanoparticles (Shanker *et al.*, 2004). Among the most important metal based Nps synthesized via the green techniques are iron, zinc, copper, gold, silver and their oxides (Younis *et al*., 2021). Currently, *Artocarpus heterophyllus* peel has been successfully used for the preparation of iron nanoparticles. Similarly, *Lansium domesticum* and *Phyllanthus emblica* fruits can synthesize gold nanoparticles with an efficient antimicrobial activity. In the industrial field, carbon nanostructures have been recently established for the production of an exceptionally photocatalytic activity and an outstanding electrical conductivity in the field of green fuels, water purification, and energy storage device (Younis *et al*.,2021)

Shahi and Patra (2003) produced nano-particles of usinic acid with an ascomycetes fungus. Ji-Hoon *et al*. (2008), produced super-paramagnetic nano-particles using *Shewanella* sp.

Yadav *et al*. (2008) produced selenium containing nano-structures using *Psuedomonas aeruginosa* while Sadowski and Maliszewska (2008) produced nano-particles of silver using *Psuedomonas strutzeri*. Senapati *et al.* (2005) produced bimetallic alloy of Au-Ag using microorganisms. Rapaid synthesis of AgNps also has been achieved using *Calendula officinalis* and *Capsicum annuum* seeds extracts. Currently, *Camellia sinensis* leaves are used in the extracellular synthesis of ZnONps as a catalytic agent (Youanis *et al*., 2021). Nowadays, there is a tremendous excitement in the study of nano-scale matter for enhancing plant productivity. However, its synthesis remains an area of primary concern if their potential is to be fully utilized (Kaul *et al*., 2012). Green synthesis has gained a great attention as a sustainable, reliable, and eco-friendly approach for the synthesis of a variety of nanomaterials.

Major drawbacks associated with the biosynthesis of NPs using bacteria are tedious purification steps and poor understanding of the mechanisms. The important challenges frequently encountered in the biosynthesis of NPs are to control the shape and size of the particles and to achieve the monodispersity in solution phase. It seems that several important technical challenges must be overcome before this green bio-based method will be a successful and competitive alternative for industrial synthesis of NPs (Iravani, 2014).

2.6 Nanomaterials as antimicrobial delivery systems

The existing disadvantages of conventional antibiotics can be solved to some extent by using nanomaterial-based antimicrobial delivery systems. In such approaches, the conventional antibiotics can be loaded into the nanoparticles through physical encapsulation, adsorption, or chemical conjugation. By this way, the pharmacokinetics and therapeutic index of the drug can

ideally be improved compared to the free form of the drug. The aimed-for advantages are provided by the improved serum solubility, prolonged systemic circulation lifetime of the drug, targeted delivery of the drug to the site of infection, sustained and controlled release of the drug, and also combinatorial drug delivery to the site of interest that could be reached by virtue of the nanoscopic delivery system (Rosenholm *et al*., 2012). This rationale of nanotherapeutics in this case aimed to enhance the therapeutic effect and minimize the side effects of antibiotics, starts with the appropriate design of nanoparticles.

In nanoparticles design, the particle size, surface properties, and the release profile of the therapeutic agent have vital impact on the success of the therapeutic approach. Lipid-based nanoparticles are widely used for the delivery of antibacterial agents. They can be designed as liposomes, solid lipid nanoparticles (SLN), and nanostructured lipid carriers (NLC). Liposomes are one of the most studied nanosystems for antimicrobial therapy in various diseases. Liposomes are spherical lipid vesicles with bilayered membrane structure, consisting of amphiphilic lipid molecules. Since their structure is similar to the bacterial cell membrane, efficacious interaction between liposomes and cells can be obtained. These interactions may create adsorption, endocytosis, lipid exchange, and fusion of the liposomes (Hallaj-Nezhadi *et al*.,2015)

The structure of liposomes, where an aqueous cavity is surrounded by lipid membranes, empowers them to transport both lipophilic and hydrophilic drugs (in lipid bilayers and aqueous compartments, respectively) without chemical modification, protecting them from degradation (Hallaj-Nezhadi *et al*., 2015). SLNs are composed of a solid lipid core stabilized by surfactants and are moderately amorphous structures in which bilayers are not distinguished. They can provide long-term stability and better incorporation efficacy for hydrophobic drugs and can be

easily scaled-up in production. NLCs were developed in order to overcome the limitations of SLNs regarding low-loading capacity for nonhydrophobic drugs and their stability issues.

In the NLC structure, liquid lipids are used to stabilize the construct, which allows a biphasic drug release profile with initial burst release continued with sustained drug release. Liposomes have shown to be successful in combating resistant pathogens. Especially, their modified designs are used to improve the potency of formulations in bacterial resistance and clearance (Yang *et al.*, 2011).

In the polymeric antibacterial drug delivery systems, drug molecules can be incorporated in the internal part of the particles, on the surface of polymeric nanocarriers with covalent or non-covalent bonds, imprinted in the polymeric nanoparticles or encapsulated in the stimuli-responsive shell of polymeric nanoparticles (Michalak *et al*., 2016).

The encapsulation route of the drug into the polymeric nanoparticle drug delivery system plays a key role in the nanocarriers' pharmacokinetic profile. The action mechanism of the polymeric nanoparticles is defined by the physicochemical properties and the composition of the particles. Polymeric nanoparticles may interact with the bacterial cell wall via passive or active targeting. Passive targeting is based on particle size and the ability of particles to disturb the structure of bacterial membrane leading to pore formation in the membrane. For active targeting of polymeric nanoparticles, the surface of polymeric nanoparticles is usually functionalized with specific antibodies and aptameric bacteriophage proteins providing specific identification for the detection of pathogens and inters- action between the particles and pathogens. To date, a significant number of reports on the activity of antibiotic-conjugated polymeric nanoparticles against various infections, including those caused by drug-resistant pathogens, have been published.

Various types of inorganic and organic nanoparticles have been utilized as antibacterial agents. (Xiong *et al*.,2014). The inherent antibacterial properties of some metals and metal oxides have been known for centuries. An important advantage of antibacterial metal and metal oxide nanoparticles is that they have multiple modes of action, which is why microbes can scarcely develop resistance to them (Karli *et al.,* 2018).

Among the inorganic antibacterial particles, silver nanoparticles are the most intensively investigated ones and capable to kill both Gram-positive and Gram-negative bacteria, having even shown to be effective against drug-resistant species. Besides silver nanoparticles, other metal nanomaterials have also been studied for antimicrobial treatment, including gold, copper, tellurium and bismuth (Karaman *et al.*, 2017). Moreover, many studies have revealed the antibacterial activity of metal oxide nanomaterials, such as zinc oxide (ZnO), copper oxide (CuO), magnesium oxide (MgO), nitric oxide (NO), titanium dioxide (TiO_2), aluminum oxide (Al_2O_3), magnetic iron oxide (α-Fe_2O_3), and cerium oxide (CeO_2) nanoparticles. The toxic mode of metal and metal oxide nanoparticles against bacterial cells has been associated with ROS generation and membrane disruption (Lemire *et al*., 2013).

2.7 Magnesium chloride

Magnesium chloride is the name for the chemical compound with the formula $MgCl_2$ and its various hydrates $MgCl_2(H_2O)x$. Hydrated magnesium chloride is the form most readily available. Anhydrous $MgCl_2$ contains 25.5% elemental magnesium by mass. These salts are typical ionic halides, being highly soluble in water. The hydrated magnesium chloride can be extracted from brine or sea water. Magnesium represents an essential element for life and is ubiquitously found in all organisms (Arbor, 1990).

This important cation plays crucial roles as an enzymatic co-factor, as well as it is involved in cellular signaling, and in stabilizing cellular components (Glasdam *et al.*, 2016). Historically, back in 1915, Professor Pierre Delbet was looking for a solution to cleanse wounds that would replace the traditional antiseptics that damage tissues. After testing several solutions, he found $MgCl_2$ solution to be most effective as it had two main advantages—it was not harmful for the tissue and it highly increased leucocyte activity and phagocytosis. Later, he found this solution to be an efficient therapy for various diseases, including diseases related to microorganisms. In the past several years, new interest on this cation arose due to its antimicrobial properties (Demishtein *et al*., 2019).

In the past several years, new interest on this cation arose due to its antimicrobial properties. In several studies, antibiotic activity in the presence of Mg^{2+} ions was found to be more efficient (Khan *et al*., 2005). It has been hypothesized that the divalent ions affect the membranes of bacterial cells. One study suggested that the curvature of the bacterial membrane is affected, and eventually the bacteria become more vulnerable, and the antibiotics are more efficient (Som *et al*., 2009). A different study by Xie *et al.* (2016) showed that these cations permeabilize the membranes and cause them to be leakier. Other studies tested the potential antimicrobial effect of coating different surfaces with magnesium or magnesium compounds. These surfaces were found as effective in prevention of bacteria adherence as well as biofilm formation. Some of these compounds were suggested to disrupt the membrane potential, again strengthening the idea that magnesium permeabilizes membranes and eventually cause the bacteria to be more sensitive (Som *et al*., 2009).

It is not surprising that magnesium salts are typically associated with positive effects on microbial cells. However, it appears that at elevated doses, for instance at millimolar

concentrations, magnesium ions become harmful for prokaryotic cell and therefore may negatively affect important cellular processes (Nguyen *et al*., 2018). Chloride (Cl-) and magnesium (Mg+2) are both essential nutrients important for normal plant growth. Magnesium and iron are essential either as structural components or as enzyme cofactors for plant metabolism. The development of nanoscale particles of Mg, Fe or their oxides may therefore help in triggering the metabolic pathways leading to better growth and higher yields of plants.

According to Konne *et al*. (2018), magnesium and calcium chlorides rank second and third respectively to sodium chloride in terms of relative abundance in seawater. Ca and Mg chlorides find applications in road construction as dust control agents, road deicing, drying agents (hygroscopic), etc. Also, comparative studies between $MgSO_4$ and $MgCl_2$ have shown that absorption and retention are more efficient with $MgCl_2$ than with $MgSO_4$ (Durlach *et al*., 2005). Chloride (C_l^-) and magnesium (Mg^{2+}) are both essential nutrients important for normal plant growth. Too much of either nutrient may harm a plant, although foliar chloride concentrations are more strongly related with foliar damage than magnesium. High concentrations of $MgCl_2$ ions in the soil may be toxic or change water relationships such that the plant cannot easily accumulate water and nutrients. Once inside the plant, chloride moves through the water-conducting system and accumulates at the margins of leaves or needles, where dieback occurs first. Leaves are weakened or killed, which can lead to the death of the tree (Jain *et al*., 2012).

2.8 Nanoparticles from magnesium

Despite of the wide application of Ag and ZnO nanoparticles, they are usually associated with high risk of toxicity due to their accumulation in the body. In contrast, magnesium is an important component required for the growth of plant. It acts as a powerhouse in the photosynthesis process. Moreover, it shows a potent interaction with plant phytoconsituents to

yield Nps. Magnesium oxide nanoparticles (MgONps) serve as a safe alternative with an extremely effective antibacterial activity as recognized by FDA (Younis *et al*., 2021). They have been used as a superior nanocarrier with unique biocompatible nature and stable physicochemical properties. They have the advantage of being highly ionic with photocatalytic characteristics and an effecient tolerance to high temperature. Recently, they have been employed as a noval application in the refractory material and as a substrate in the biomedical field. Despite of their promising antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis*,and *Pseudomonas aeruginosa* (Hassan, 2021).

Moreover, metal oxide nanoparticles of MgO were tested as antibacterial agents as well (Nguyen *et al*., 2008). Indeed, these particles were found to be effective against yeast and planktonic bacteria as well as against biofilms. In addition, these nanoparticles were found to be of low cytotoxicity and relatively safe. Since biofilm formation is considered as a major problem in the food industry as well as in the biomedical field, a lot of effort is put into dealing with this phenomenon (Alvarez-Ordonez *et al.,* 2019).

2.9 Antimicrobial Activity of Magnesium Nanoparticles

Bacterial contamination continues to draw public attention. Therefore, in order to solve this problem, it is highly necessary to develop effective antimicrobial agents to control the bacterial population (Kumar *et al*., 2008). Generally, antibacterial agents can be categorized as organic or inorganic antibacterial agents. Organic antibacterial agents such as organic acids, essential oils, bacteriocins and enzymes have been widely studied. However, they have some shortcomings, such as low resistance to processing conditions, which limit their applications. As a result, inorganic antibacterial agents have attracted much interest for bacterial control (Jung *et al*.,

2008). The main advantages of inorganic antibacterial agents, compared to organic antibacterial agents, are the improved stability under harsh processing conditions.

Presently, some of the inorganic antibacterial materials, in particular inorganic metal oxides such as TiO_2, ZnO, MgO and CaO, have been studied (Huang *et al*., 2000). Among the studied inorganic metal oxides, ZnO, MgO and CaO are of particular interest because they are not only stable under harsh process conditions, but also generally regarded as safe materials to human beings (Stiomenov *et al*., 2002). Additionally, they have antimicrobial activity without photo-activation, compared to TiO2 that requires photo-activation (Stiomenov *et al*., 2002).

According to Tang and Lv (2014), in recent times, nanosciences and nanotechnology has been leading to a technological revolution in the world, which is concerned with materials with significantly novel and improved physical, chemical and biological properties. Nanoparticles are recognized as antibacterial agents due to their size, structure, and surface properties. Thus, nanotechnology offers a way to improve the activity of inorganic antibacterial agents. Metal oxide nano particles such as ZnO, MgO and CaO have been investigated as inorganic antibacterial agents (Stiomenov *et al*., 2002).

2.10. Advantages of nanotechnology-based antimicrobial delivery

Administration of antimicrobial agents using nanoparticles can increase the overall pharmacokinetics by progressing therapeutic index, extending drug circulation, and providing controlled drug release. Multiple mechanisms of action can be provided by the antibacterial nanoparticles, which prevent the development of antibacterial resistance by many pathogenic bacteria. Several routes of administration, including oral, nasal, parenteral, intraocular, and so on, can be employed with the nanotechnology-based antibacterial treatments.

With the use of nano-carriers in the delivery of drugs, combination drug therapy can be achieved by incorporating two or more drugs or different therapeutic modalities into the carrier matrix. The surface modifications can be carried out by conjugating targeting ligands on the nanocarriers that are not known by the immune system and specifically targeted to special microorganisms. The significant advantages of nanomaterials as antimicrobial agents are their modularity in design, enabling a multimodal approach that makes it especially difficult for bacteria to develop resistance mechanisms against these. A nanotechnology-based antibacterial agent can be constructed out of several components that possess antimicrobial activities in themselves, such as, for instance, be composed of an antibacterial core material (e.g. metal or metal oxide) surrounded with an antibacterial polymeric shell or coating, in which antibiotic drugs could be incorporated (Lam *et al*., 2016). The core material could further be "prickly," which physically can destroy the bacterial cell wall by a "nano-piercing" process once the polymeric shell has been dissolved, leading to the disruption of bacterial integrity and lysis, as presented in a recent study by Wu *et al*. (2016), where zinc-doped copper oxide prickly nanoparticles exhibited high bacterial killing efficiency owing to the provided core particle nanostructure.

Furthermore, varying possibilities for combination therapy together with existing (commercial) antibiotics to reach synergistic effects are evident (Smekalova *et al*., 2016).

2.11 Challenges of nanotechnology-based antibacterial treatments

Although nanoparticle-based antibacterial treatments promise significant benefits and advances in addressing the key hurdles in treating infectious diseases, there are challenges in translating this exciting technology for clinical use. These include thoroughly evaluating the interactions of nanoparticles with cells, tissues, and organs, which accordingly recalibrates doses and identifies proper administration routes to obtain desired therapeutic effects. Hence, to provide a clinical

translation of nanomaterials, standardized in vitro experimentations that will provide in vivo relevant data should be established (Huh and Kwon, 2011). However, according to Karaman *et al*. (2017), the absence of standardizations in testing methods leads to inconsistency in results. The foremost requirement of the assays applied to the evaluation of antimicrobial activity is reproducibility. Antimicrobial activity should be tested against various microorganisms, preferably against representatives of both Gram-negative and Gram-positive species. Moreover, a combination of several assays is preferred to confirm the activity (Karaman *et al.,*2017).

2.12 Plant Disease

A plant disease is any physiological or structural abnormality that is caused by a living organism. Organisms that cause diseased are referred to as 'pathogens', and affected plants are referred to as 'hosts'. Many organisms rely on other species for sources of nutrients or as a means of survival but are not always harmful to the hosts. Plant pathogen on the other hand, utilizes hosts for nutrients and/or reproduction at the host's expense. Disease causing organisms include Fungi, Molds, bacteria, viruses, nematodes, phytoplasmas, and parasitic seed plants (Waleed *et al.,*2016).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Collection of Diseased Plant Sample

Diseased plant sample were collected from three different farms in Ugbowo, Benin city**,** Edo state. The samples were collected into sterile sample polythene bags with proper labeling and thereafter transported to the laboratory for analysis.

3.2 Sterilization of Materials

Glasswares like test tubes, beakers, measuring cylinder, conical flask, pipette tips needed to carry out this project were soaked and washed in detergent and rinsed with distilled water. They were wrapped with aluminum foil paper and autoclaved in an inverted position at 160-170^{o}C for 45-50 minutes.

3.3 Preparation of Nutrient Agar

2.8g of nutrient agar powder was dissolved in 100ml of distilled water in a conical flask and covered thoroughly with Aluminum foil paper. 100mg of ketoconazole was added into the already prepared nutrient agar to prevent the accrual of Fungi(0.1g). This was stirred and autoclaved at 121^{o}C for 15minutes and then cooled to 45-50^{o}C. A portion of the Medium (20ml) was poured into five (5) sterile petri dish and allowed to solidify**.**

3.4 Isolation of Bacteria Phytopathogen from the Diseased Cassava Plant

A small piece of diseased plant was acquired sterile from the diseased plant sample using direct method of inoculation. It was rinsed in distilled water afterwards 5ml of 3% sodium hypochlorite was prepared using the formula $C_1V_1=C_2V_2$ to calculate for the volume of the diluent required where $C_1=3.5$, $V_1=?$, $C_2=3$ and $V_2=5ml$

$V_1=C_2V_2/C_1=3X5/3.5= 15/3.5$

$V_1=4.28$

5ml - 4.28=0.72ml

0.72ml of distilled water was added to 4.28ml 3.5% of sodium hypochlorite which was used to sterilized the surface of the diseased cassava leaf after which it was immersed in sterilized distilled water and then was picked appropriately with a sterilized forceps and inoculated on the solidified agar plate and was incubated for the next 24hours. All process was duly carried out under aseptic conditions.

3.5 Identification of Bacteria Isolates

After incubation for 24hours, the number of colonies were counted and growth was observed in three plates. The bacteria isolates were identified on the biochemical level by carrying out series of biochemical tests such as Gram staining, Catalase test, Motility test, Starch hydrolysis test, Methyl red test and Thioglycolate test. The bacteria isolates were equally identified molecularly by extraction of the bacteria DNA.

3.5.1 Gram Staining

An already prepared sample was inoculated on a clean slide to make a smear. After which it was allowed to air dry and then heat fixed by passing the slide carefully through a bursen flame. Then it was flooded with the primary stain (crystal violet) for 60seconds and then rinsed carefully with water. Next it was flooded with iodine which is a mordant and allowed to stand for 60seconds and then rinsed with water. Then the sample was flooded with alcohol for 3seconds to decolorize the sample and rinsed with a gentle stream of water. Lastly safranin was added to the slide for

60seconds and again rinsed with a gentle stream of water after which it was allowed to air dry and viewed under a microscope with a drop of immersion oil (Mittwer et al., 1992)

If the bacteria are stained purple, it is a gram-positive bacterium,

If the bacteria are stained pink, it is gram-negative bacterium

3.5.2 Catalase Test

A Microscopic slide was placed in a petri dish so as to reduce catalase aerosols. Then a small amount of organism was inoculated from a well isolated 24-48hrs colony with a sterile inoculating loop and placed on to a microscopic slide. Then a drop of 3% H_2O_2 was added to the microscopic slide containing the organism using a dropper pipette and was observed for bubbles under a black background to enhance readability (Khatoon *et al*., 2022).

A positive test was confirmed by immediate formation of bubbles,

A negative test is represented by no bubbles

3.5.3 Motility Test

1.7_g of nutrient broth was dissolved in 95ml of distilled water and autoclaved. An equal amount of the broth was inoculated into sterile tubes and allowed to cool. Using a sterile loop, a swab of the bacteria was obtained from each agar plate containing pure culture and inoculated into the tubes containing nutrient broth and was incubated for 24hrs before observing results (Acharya, T., 2022). If the bacteria spreads on the surface of the nutrient broth, it is motile, If the bacteria do not spread on the surface of the nutrient broth, it is non-motile.

3.5.4 Starch Hydrolysis Test

Using a single streak inoculation technique, the sample organism was streaked on the agar plate and incubated for 24hrs at 37^{o}C. After the incubation period the medium was flooded with iodine solution and observed for result (Sigmon, 2008).

A clear zone around the organism which indicated that starch was degraded (positive) and no clear zone which indicated that starch was not degraded (negative).

3.5.5 Thioglycolate Test

1.71$_{g}$ of nutrient broth was autoclaved after being dissolved in 95ml of distilled water. The broth was inoculated into sterile tubes in equal parts and allowed to cool. Using a sterile loop, a swab of bacteria was taken from the agar plates and inoculated into tubes containing nutrient broth, which were then inoculated for 24hrs before the results were obtained.

The presence of bacteria at the bottom of the broth indicates that the bacteria is anaerobic. However, if growth appears on the surface it is aerobic.

3.6. Molecular Identification

STEP 1: Using a sterile loop, bacteria cell was inoculated into 200µl of nuclease-free water using a micropipette into a centrifuge tube aseptically

STEP 2: Bacteria cells that have been re--suspended in 200µl nuclease-free water were added to a ZR Bashing Bead Lysis Tube

STEP 3: 750µl of Bashing Bead buffer was inoculated into each Bashing Bead tube using a sterile micropipette which served as a lysing solution

STEP 4: Each tube was placed in a thermomixer for 8 minutes at 60^{o}C

STEP 5: Centrifugation was done at 10,000 × g for 60 seconds for the purpose of separating solid and liquid particles on the principle of gravitational forces.

STEP 6: 400µl supernatant was transferred to a Zymo-Spin III-F filter in a collection tube and centrifugation was done at 8,000× g for 60 seconds.

STEP 7: 1200µl of genomic lysis buffer was added to the filterate in the collection tube.

STEP 8: 800µl of the mixture was transferred to a Zymo-Spin IICR Column[3] in a collection tube and centrifugation was done at 10,000 × g for 60 seconds. The flow through was discarded from the collection tube and the process was repeated

STEP 9: 200µl of DNA prewashed buffer was added to the Zymo-Spin IICR Column in a new collection tube and recentrifuged at 10,000×g for 60 seconds, the was buffer helps to purify the DNA.

STEP 10: 500µl of DNA wash buffer was added to the Zymo-Spin IICR column and centrifuged at 10,000×g for 60 seconds.

STEP 11: Zymo-Spin IICR Column was transferred to a clean 1.5ml micro centrifuge tube. 70µl of DNA elution was added directly to the column matrix and centrifuged, at 10,000×g for 30 seconds. Check next chapter for results

3.7 Preparation of *Moringa oleifera* Aqueous Extract

The aqueous leaf extract of *Moringa oleifera*, previously obtained from three different farms in Ugbowo, Benin City, Edo state was prepared by weighing 20g of the fresh leaves and washing with distilled water followed by 70% ethanol to sterilize the surface for about 120 seconds and then washed again with distilled water after which it was mashed and transferred into a beaker

containing 100ml of sterile distilled water and the mixture was boiled for 10 minutes (Igiehon *et al.*, 2020).

3.8 Synthesis of Magnesium Chloride Nanoparticles

2.3g of $Mgcl_2$ nanoparticles was dissolved in 113ml of sterilized water then 38ml of plant extract was added after which an aliquot of 10ml was measured into seven (7) test tubes and incubated at room temperature. A dark-brown appearance indicated the formation of magnesium chloride Nanoparticles ($Mgcl_2$NPS) (Shittu *et al*.,2020).

3.9 Characterization of Magnesium Chloride Nanoparticles

The solution was monitored using UV-visible spectrophotometer to identify and characterized the synthesized the nanoparticle quantitatively. This was carried out by measuring the absorbance at regular intervals (2hrs and 24hrs after synthesis) within the wavelength of 300-500nm (Igiehon *et al.,*2020)

3.10 Antimicrobial Effects of Magnesium Chloride Nanoparticles on Bacteria Phythopathogen from the Diseased Cassava.

Magnesium chloride nanoparticles synthesized from *Moringa oleifera* leaf extract and magnesium chloride solution were used in different concentration. The different concentration corresponding to 100%, 50%, 25% of Magnesium chloride nanoparticle was used. The suspension of the organism from the pure culture was compared with that of Macfarland's solution after which 1ml of the suspension was added to each labeled Petri dish accordingly. The prepared agar was added to the Petri dish and rocked then allowed to solidify. After the agar had solidified wells were bored using a sterilized borer according to the alphabet A-F. Antibiotics was added to well A, Plant extract to well B, precursor to well C, 25% nanoparticle to well D,

50% nanoparticles to well E and 100% nanoparticles to well F. Each petri dish was then sealed. Zones of inhibition were measured afterwards in cm.

CHAPTER FOUR

The phytopathogenic bacteria isolated and identified were *Enterobacter hormaechei* S36-2, *Enterobacter ludwigii* Gm7 and *Erwinia* spp

The morphological and the result for the biochemical test are shown in the plates and table below.

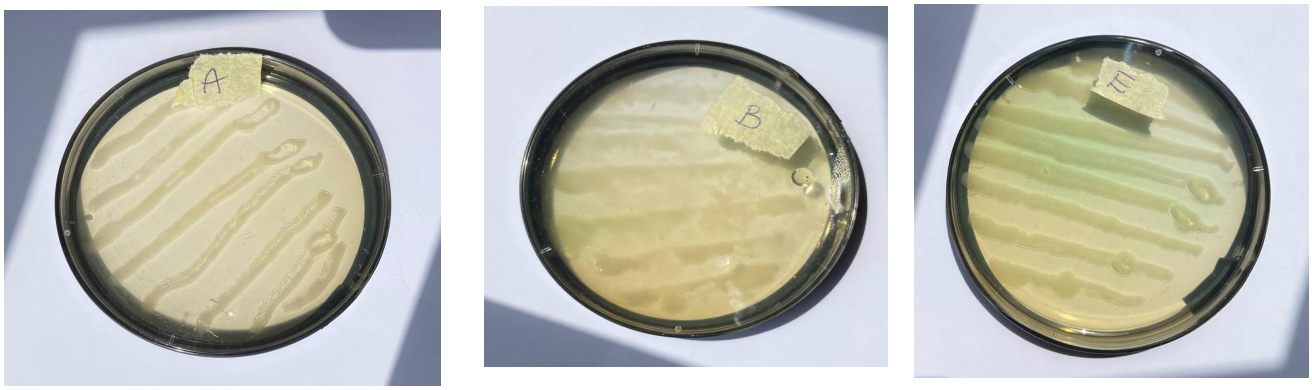


PLATE 1: Pure culture of the bacteria isolated from diseased *Manihot esculenta* leaf

A: *Enterobacter hormaechei* S36-2

B*: Erwinia spp*

C: Enterobacter Ludwigii GM 7

TABLE 1: Morphological and biochemical test of bacteria isolated from diseased cassava plant.

Isolate	1	2	3
Elevation	Raised	Convex	Raised
Margin	Entire	Entire	Entire
Color	Cream	Cream	White
Shape	Circular	Irregular	Circular
Size	Medium	Small	Small
Gr. Diff.	MCA	NA	MCA
Morphological			
Gram stain	-	-	-
Cell type	Rod	Rod	Rod
Arrangement	Dispersed	Dispersed	Dispersed
Color	Pink	Pink	Pink
Biochemical Test			
KOH	-	+	-
Indole	-	-	-
Catalase	+	-	+
Citrate	+	+	+
Oxidase	-	+	-
Glucose	+	+	+
Sucrose	+	+	+
Lactose	+	-	+
Mannitol	+	+	+
Gas formation	+	+	-
Motility	+	+	+
H_2S formation	-	+	-
Identity	*Enterobacter hormaechei* S36-2	*Erwinia* spp	*Enterobacter Ludwigii* GM7

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The bacterial isolate 1 has similar sequence with *Enterobacter hormaechei* strain S36-2 with accession number MT337458.

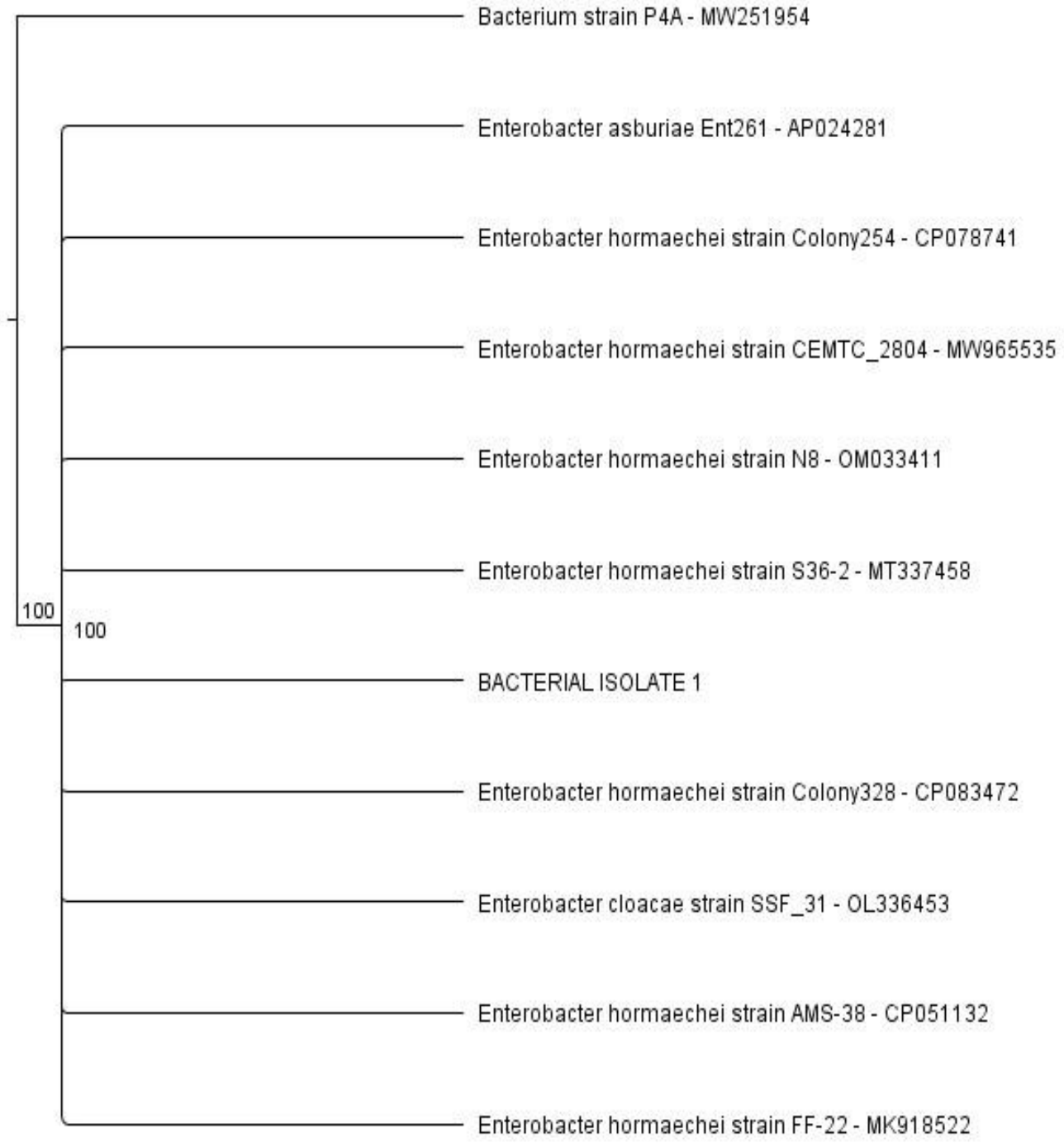


FIGURE 1: Phylogenetic analysis of isolate 1 based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of bacteria.

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The bacterial isolate 2 has similar sequence with *Enterobacter ludwigii* strain GM7 with accession number KR002425.

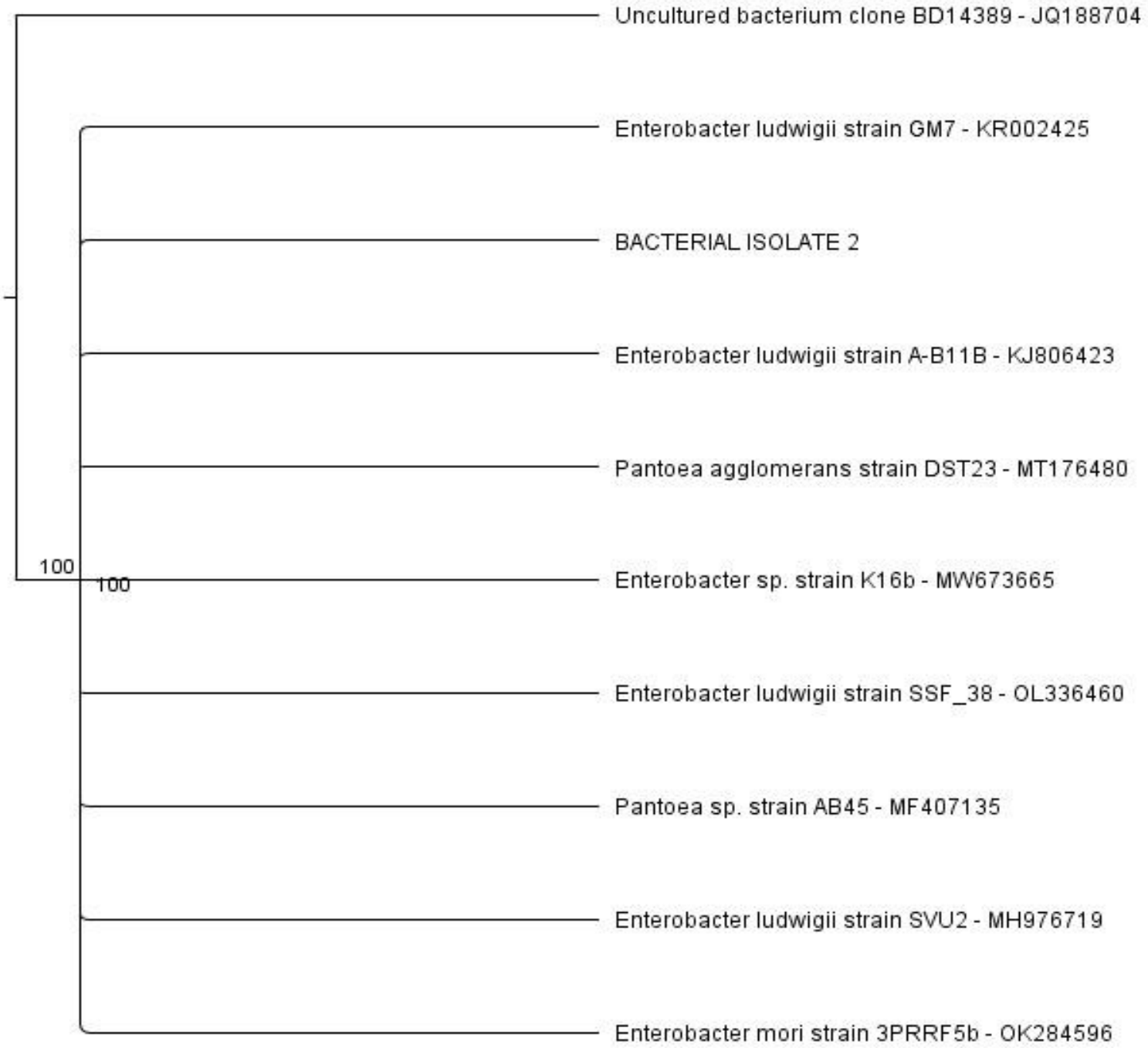


FIGURE 2: Phylogenetic analysis of isolate 2 based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of bacteria.

PLATE 2: Materials used for magnesium chloride nanoparticles synthesis

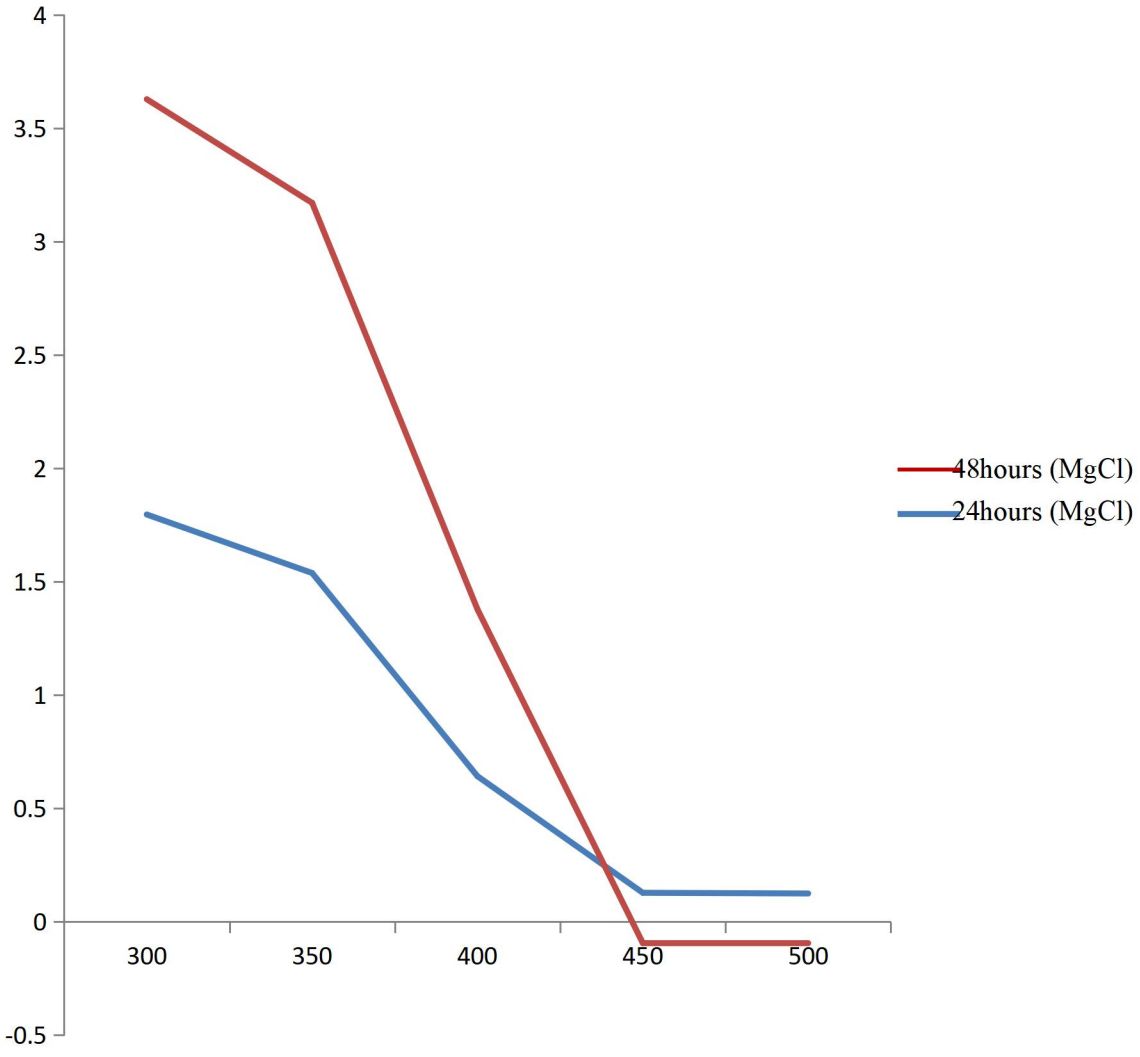
A:*Moringa oleifera* plant extract

B: Magnesium chloride precursor

C: Magnesium chloride nanoparticles

UV-VIS SPECTROPHOTOMETRIC ANALYSIS

The Spectrophotometric result indicated that the absorbance values decreased with time (in hours) of synthesis. The maximum absorbance values was recorded at 350nm $Mgcl_2$ Nps



Absorbance

Figure 3: UV Spectrophotometric reading on $Mgcl_2$ nanoparticles

TABLE 2: Color description of *Moringa oleifera*, plant extract, $Mgcl_2$ precursor and the nanoparticles synthesized using color namer

DESCRIPTION	PLANT EXTRACT	PRECURSOR	NANOPARTICLES
RGB	95.77,34	176,180,196	93,6517
COLOR	Dark brown	Ash grey	Sepia(Brown family)

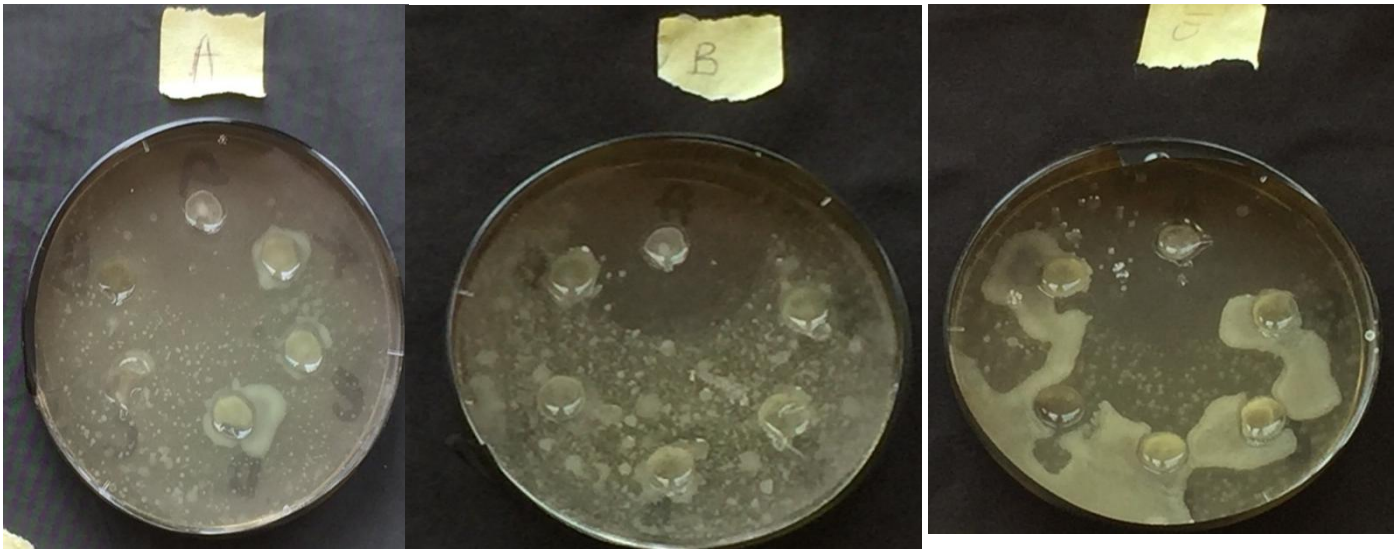


PLATE 3: Agar well showing inhibition by Antibiotics (A) on Plate A,B and C

TABLE 3: Antibacterial activity of $Mgcl_2$ nanoparticle 24hrs after incubation against selected bacterial pathogen represented by the zone of inhibition(cm).

TEST ORGANISM	CONCENTRATION (%)			CONTROLS		
	25	50	100	$Mgcl_2$ Precursor	*Moringa oleifera* leaf extract	Chloramphenicol
Enterobacter hormaechei S36-2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.76±0.02a
Erwinia sp	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.20±0.10c
Enterobacter Ludwigii GM 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.60±0.10a

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance

TABLE 4: Antibacterial activity of $Mgcl_2$ nanoparticles 48hrs after incubation against selected bacterial pathogen represented by the zone of inhibition(cm)

TEST ORGANISM	CONCENTRATION (%)			CONTROLS		
	25	50	100	$Mgcl_2$ Precursor	*Moringa oleifera* leaf extract	Chloramphenicol
Enterobacter hormaechei S36-2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.60±0.10a
Erwinia sp	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.20±0.10c
Enterobacter Ludwigii GM 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.76±0.02a

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance

CHAPTER FIVE

DISCUSSION

This study was to evaluate the effect of incubation period on the antimicrobial activities of magnesium chloride nanoparticles on phytopathogens isolated from a diseased *Manihot esculenta* leaf. Magnesium chloride nanoparticles were synthesized biologically using *Moringa oleifera* and the results were in line with those described by Sharma *et al.(*2017) and Archana *et al*. (2021) it was reported that when the aqueous Mgcl were combined with *Moringa oleifera* extract the Mg+ was reduced to its nanoparticle form resulting in a color change from Grey to Sepia brown.

The prepared magnesium chloride nanoparticle was characterized using spectroscopic technique. The UV-vis absorbance spectroscopy revealed the optical properties and values of Magnesium Chloride nanoparticles, the peak value observed was 350nm which is in line with an earlier study by Sutapa *et al*. (2018).

The antibacterial activity of magnesium chloride nanoparticles of *Moringa oleifera* extract was evaluated against plant pathogenic bacteria, *E.hormaechei* S36-2, *Erwinia* spp and *E. ludwigii* GM7, the magnesium chloride nanoparticles displayed no activity against the test organism at all tested concentrations (25%,50% and 100%). However, the test organisms were susceptible to standard antibiotic, Chloramphenicol, with zones of inhibition ranging from 2.60 ± 0.10cm to 3.20 ± 0.10cm.

Contrary to this study, Fatiqin *et al*. (2021) reported that magnesium oxide nanoparticles of *Moringa oleifera* extract had antibacterial activity against *S. aureus* and *E. coli* with the inhibition zones of 6.30cm and 6.00cm. Similar studies by Amrulloh *et al*.,2021 observed MIC

values which ranged from 300-550ug/mL against both Gram positive (*S. aureus* and *E. faecalis*) and Gram negative (*E. Coli* and *S. dysenteriae*).

The inactivity of the magnesium chloride nanoparticles used in this study are in sync with the reports by Sorbium *et al*. (2018) who had similar results and opined that ineffectiveness of nanoparticles could be as a result of size owing to the fact that nanoparticles larger than 10nm may not interact with the bacteria and may not produce electronic effects which improve the reactivity of nanoparticles. This also correlate with the submissions of Hassan *et al*. (2016) who reported no antifungal activity of silver nanoparticles against *Rhizopus stolonifera* at all tested concentration.

Conclusion

The result of this study reveals that magnesium chloride nanoparticles (at all concentration) had no effect on the pathogens. Further studies should be however conducted to unravel the reasons behind this resistance by the test isolates against synthesized magnesium chloride nanoparticle.

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