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ISOLATION AND MOLECULAR IDENTIFICATION OF SOME TYPES OF SOIL BACTERIA, TESTING ITS ABILITY TO PRODUCE SILVER NANOPARTICLES AND INVESTIGATING THEIR EFFECTIVENESS AGAINST *LEISHMANIA TROPICA* AND MOLECULAR ANALYSIS OF THIS PARASITE

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November 2022

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ABSTRACT

ISOLATION AND MOLECULAR IDENTIFICATION OF SOME TYPES OF SOIL BACTERIA, TESTING ITS ABILITY TO PRODUCE SILVER NANOPARTICLES AND INVESTIGATING THEIR EFFECTIVENESS AGAINST *LEISHMANIA TROPICA* AND MOLECULAR ANALYSIS OF THIS PARASITE

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Master of Science in Biology

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November 2022

This study was designed to synthesize AgNPs from supernatant-free cellcultures of some soil bacteria. Three bacteria (*Pseudomonas aeruginosa* (strain ALK320), *Bacillus cereus* (strain HNB5), and *Corynebacterium striatum (*strain 1910I14)) have been isolated and confirmedby PCR with universal primers. Then, the sequence results aligned with National Center for Biotechnology Information (NCBI) to ensure the species. These three bacteria were grown in nutrient broth to produce nanoparticles. Many approachesareusedtoverifytheproduction of AgNPs. Theystartwithcolorchanges, UV-visible, TEM, Scanning Electron Microscope, and end with EDX. The polymerase chain reaction (PCR) was used to identify *Leishmania tropica (L. tropica)*; in which two primers were used to amplifythe *18sRNA* gene, and they are a specific primer of gene LITSR/L5.8S, which gave an amplification product of 450bp, and a specific primer of gene mkDNA, which provided an amplification product of 120bp. The sequencing results showed a matchof 100% for *L.tropica* in the NCBI using the Basic Local Alignment Search Tool (BLAST). The maximum antileishmanial activity of extracellular AgNPs on *L. tropica* is recorded as growth inhibition (64.72%)at 400 µL /mL after being suppressed following 24 h of incubation by AgNPs synthesized using *P. aeruginosa*, *B. Cereus*and *C. Striatum*assays.The MTT assay revealed incredible results for AgNPs as *Leishmania* antiparasitic activity, to sum up, our novel approach to

leishmaniasis treatment opens up new possibilities. Also, these AgNPs synthesized from natural materials can be used to treat a wide range of pathogenic bacteria and parasites.

2022, 73 pages

Keywords: AgNPs, Soil bacteria, DNA extraction, Sequencing, *Leishmania tropica*

ÖZET

BAZI TOPRAK BAKTERİ TÜRLERİNİN İZOLASYONU VE MOLEKÜLER TANIMLANMASI, GÜMÜŞ NANOPARTİKÜLLER ÜRETME YETENEKLERİNİN TEST EDİLMESİ VE *LEİSHMANİA TROPİCA*'YA KARŞI ETKİNLİKLERİNİN İNCELENMESİ VE BU PARAZİTİN MOLEKÜLER ÇALIŞMASI

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Kasım 2022

Bu çalışma, bazı toprak bakterilerinin süpernatant içermeyen hücre kültüründen AgNP'leri sentezlemek için tasarlanmıştır.Üçbakteri (*Pseudomonas aeruginosa* (suşu ALK320)*, Bacillus cereus* (suşu HNB5)ve *Corynebacterium striatum* (suşu 1910I14) evrensel primerlerle polimeraz zincir reaksiyonu (PCR) ile izole edilmiş ve doğrulanmıştır. Ardından, dizi sonuçları, türleri doğrulamak için Ulusal Biyoteknoloji Bilgi Merkezi (NCBI) ile hizalanmıştır. Bu üç bakteri, nanopartiküller üretmek için besin suyunda büyütülmüştür. AgNP'lerin üretimini doğrulamak için kullanılan birçok yaklaşım, renk değişiklikleri, UV-görünür, TEM, Taramalı Elektron Mikroskobu ile başlar ve EDX ile biter. *L. tropica'yı* tanımlamak için PCR kullanılmıştır. Burada *18sRNA* genini amplifiye etmek için iki primer kullanılmıştır. Bunlar, 450bp'lik bir amplifikasyon ürünü veren LITSR/L5.8S geninin spesifik bir primeri ve 120bp'lik bir amplifikasyon ürünü veren spesifik bir gen mkDNA primeridir. Sıralama sonuçları, Temel Yerel Hizalama Arama Aracını (BLAST) kullanarak NCBI'da *L. Tropica* ile %100 bir eşleşme gösterdi. *L. Tropica* üzerindeki ekstraselüler AgNP'lerin maksimum antileishmanial aktivitesi, sırasıyla *P. aeruginosa*, *B. Cereus*ve *C. Striatum* kullanılarak sentezlenen AgNP'ler tarafından 24 saatlik inkübasyondan sonar baskılandıktan sonra 400 µL /mL'de % 64,72 ile büyüme inhibisyonu olarak kaydedilir. Şaşırtıcı bir şekilde, MTT tahlili, *Leishmania* antiparaziter aktivitesi olarak AgNP'ler için inanılmaz sonuçlar ortaya koydu. Özetleme kgerekirse, leishmaniasis tedavisine yönelik yeni yaklaşımımız

yeni olanaklar sunuyor. Ayrıca, doğal kaynaklardan sentezlenen bu AgNP'ler, çeşitli patojenik bakteri ve parazitleri tedavi etmek için kullanılabilir.

2022, 73 sayfa

Anahtar Kelimeler: AgNPs,Toprak bakterileri, DNA ekstraksiyonu, Dizileme, *Leishmania tropica*

PREFACE AND ACKNOWLEDGEMENTS

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Mohammed Atiyah Ghanim AL-MARYANI
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LIST OF SYMBOLS

%	Percent
°C	Degrees celsius
Bp	Base pair
KNO_3	Potassium nitrate
mg	Milligram
mL	Milliliters
nm	Nanometer
rpm	Rotation per minute
ug	Microgram
µL	Microliter

LIST OF ABBREVIATIONS

AgNO3	Silver nitrate
AgNps	Silver nanoparticles
APE	Acid phosphatase enzymes
BLAST	Basic local alignment search tool
CP	Cysteine protease
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDX	Energy dispersive x-ray spectroscopy
ELISA	Enzyme linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
L	Leishmania
LPG	Lipophosphoglycan
MLEE	Multilocus enzyme electrophoresis
NCBI	National center for biotechnology information
NNN	Novy-MacNeal-Nicolle
NPs	Nanoparticles
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic
RFLP	Restriction fragment length polymorphisms
ROS	Reactive oxygen species
RPMI 1640	Roswell park medium
STM	Scanning tunneling microscope
TEM	Transmission electron microscopy

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1. INTRODUCTION

Molecular biology is the study of the molecular foundations of biological processes in living organisms including the understanding of the interactions between biomolecules, which constitute the basis of life, as well as the control of these interactions. The nucleic acids (DNA/ RNA), proteins, and their products are all important in molecular biology. Nucleic acids are the essential elements of a living cell and encode and convey genetic information required for the survival of all tissues and organisms (Craig *et al*. 2021).

Numerous researchers studying in the field of Nano medicine are interested in fighting against multidrug-resistant illnesses. *Leishmaniasis* is a dangerous illness that affects around 12 million people annually in some 98 countries worldwide, with an estimated 350 million more at risk of developing it. Moreover, 1.5 million people contract cutaneous leishmaniasis, whereas 500,000 contract visceral leishmaniasis every year as well. The above stated, leishmaniasis cases are on the rise due to a spike in disease vectors caused by global warming (Palma *et al.* 2021).

Leishmaniasis is classified as cutaneous, mucocutaneous, diffuse cutaneous, and visceral patterns, each of which has its own set of clinical symptoms (Marquardt *et al*. 2000, Fida *et al.* 2021). Conventional diagnostic methods, which lacked sensitivity and specificity and didn't show evidence about pathogenic species, were replaced by the polymerase chain reaction (PCR).

Pentavalentantimonial compounds are among the various drugs used to treat leishmaniasis and are regarded as gold standard, which is proven to be highly toxic to humans. *Leishmania* parasites have been sometimes found to be resistant to antileishmanial medicine(Natera *et al.* 2007). Another disadvantage is that medications are ineffective against many strains of *Leishmania*. Hence, new treatment methods for leishmaniasis are urgently needed (Alemzadeh *et al*. 2022).

Because of their strong reactivity to chemicals, nanoparticles, in particular, are capable of creating reactive oxygen species (ROS), which can eliminate harmful bacteria. They found that NPs cause infections by altering the structure of lipophosphoglycan and glycoprotein molecules on the surface of parasites (Sirelkhatim *et al*. 2015).

The researchers state that nanoparticles may enter the cell directly via cell membrane pores or indirectly through ion channels and transporter proteins on the plasma membrane. Nanoparticles enter cells via endocytosis process. Nanoparticles injected into the cell might directly interact with oxidative organelles such as mitochondria. Furthermore, Ag ions bind with enzyme disulfide or sulfhydryl groups, interrupting metabolic pathways and eventually leading to cell death (Adibkia*et al*. 2010).

1.1 Aim of Study

The aim of this study is to select and improve bacterial strains isolated from soil potential extracellular biosynthesis of silver nanoparticles (AgNPs), evaluate the activity of AgNPs, synthesized in the laboratory as anti-*Leishmaniatropica*, provide an accurate physical and biological description for these particles, to know their characteristics, and distinguish between them, *16SrRNA* was used to figure out which types of bacteria were taken from the soil to make nanoparticles by comparing them with the Gene Bank in Korea and the National Center for Biotechnology Information.

In this study, *Pseudomonas aeruginosa* strain ALK320, *Bacillus cereus* strain HNB5, and *Corynebacterium striatum* strain 1910ICU141 from the soil were grown in a laboratory environment and their cell-free supernatants were used to make AgNPs.

2. LITERATURE REVIEW

2.1 History of Molecular Biology

Asbury, a British physicist, introduced molecular biology in 1945. Moreover, the scientific community did not recognize it until the 1970s. A complex system or an advantageous approach could be made straightforwardly by using bacteria and bacteriophages. This is because these organisms yield information about basic biological processes more readily than animal cells. Following Rosalind Franklin and Maurice Wilkins' work, Francis Crick and James Watson showed the DNA structure in 1953 at the Medical Research Council's Cavendish laboratory in Cambridge. Surprisingly, their findings identified DNA material in other microorganisms, plants, and animals.Molecular biology refers to the study of biological molecules, how they interact with one another and various methods developed.Moreover, one of such techniques that has a significant impact is the PCR, which was invented in 1983 and has been widely used in biology.Additionally, PCR has been applied in various fields of biology, ranging from prokaryotes to eukaryotes (Morange 2001).

2.1 Basic Molecular Biology Techniques

2.1.1 Enzymes used in molecular biology

The discovery and characterizations of several enzymes have aid numerous DNA analyses and manipulation methods that can recognized the DNA sequences and DNA cleavage. One of these enzymes is type II restriction endonuclease, which has become that can recognized the DNA sequences and DNA cleavage indispensable for molecular biologists. These enzymes are capable of recognizing certain DNA sequences and cleaving the DNA.

2.1.2 Isolation and separation of nucleic acid (DNA)

DNA is usually separated and purified before it is analyzed or manipulated. This method of cell rupture is the gentlest possible to avoid fracturing DNA due to mechanical shearing. In addition, DNase enzymes require Mg_{21} ions, which EDTA can chelate during this process. Enzymatic digestion (for example, using bacteria treated with lysozyme) and destruction of cell walls and membranes. Cell disruption should be performed at 4 °C using autoclaved equipment and solutions.

2.1.3 Electrophoresis of nucleic acids

The most popular technique for separating DNA molecules depending on their size is electrophoresis on agarose or polyacrylamide gels. The approach might be analytical or preparatory and qualitative or quantitative. The pulsed field gel electrophoresis (PFGE) method may be used to separate chromosomes. The most frequent and easiest form of electrophoresis is ethidium bromide staining on horizontal agarose gels. When this dye is exposed to UV light and binds to DNA by insertion between stacking base pairs, it emits an orange or red fluorescence (intercalation). During DNA cloning operations, electrophoresis is routinely employed to check the purity and integrity of a DNA preparation or to assess the extent of an enzyme reaction (Rapley and Whitehouse 2015).

2.1.4 Nucleic acid analysis methods

There are many techniques are available to analyzing DNA and RNA, but a large number of them depend on solution or more, chip-based array devices to accomplish their goals. Recent advancements in this field indicate that many detections and analyses methods may be implemented in this format.

2.1.5 Gene probe derivation

Many molecular biology procedures necessitate the availability of a gene probe, even though this is often one of the most difficult steps to complete.Gene probe data can be obtained from various sources.However, upon the advancement and sophistication of genetic databases, this is frequently one of the first steps in the production of gene probes.There are several genetic databases situated across the globe. These databases may be searched using the internet to uncover precise sequences linked to a certain gene or protein of interest. In certain circumstances, closely similar proteins from the same gene family to get information on the most beneficial DNA sequence have been utilized(Rapley and Whitehouse 2015).

2.1.6 The polymerase chain reaction (PCR)

In recent years, there have been several notable advances in molecular biology methods. PCR is often regarded as the quickest and most straightforward method of amplifying any piece of DNA. Simply put, the PCR has had the greatest impact in recent years for two reasons:it is extremely easy to use and relatively ease to complete the actual manipulation stages. Rapley and Whitehouse (2015), described the process as amplifying a specific DNA fragment and eliminating the need for more time-consuming and labor-intensive cloning procedures. The use of PCR technique in diagnosing many pathogenic microorganisms has become a standard practice for more than a decade. The PCR technique has also been extensively used to document the *Leishmaniaparasite* (Rapley and Whitehouse 2015).

2.2 Nanotechnology

In 1974, Norio Taniguchi (Tokyo Science University) coined the term nanotechnology, which means "Nano scale technology". Nanotechnology refers to the processing, separation, consolidation, and deformation of materials carried out by a single atom or molecule.The invention of the scanning tunneling microscope and the development of

cluster science were two significant discoveries in the early 1980s when nanotechnology and Nano science (STM) boosted, resulting in discovery of fullerenes in 1985. Nanoparticles have been utilized in a variety of scientific fields. Furthermore, nanoparticles are used in the diagnosis, treatment, and prevention of a wide range of diseases. An interesting development in the antimicrobial field is the development of a new strategy, nanotechnology, to fight against the spread of antibiotic-resistant bacteria(Rai *et al*. 2012).

2.2.1 Silver nanoparticles

Silver has been recognized and used for its medicinal and therapeutic properties long before discovering microorganisms as infectious agents and for much longer than the recorded history. Additionally, it is available in various shapes and sizes, including solutions, foils, sutures, and collides such as lotions and ointments. Treating infectious disorders and surgical infections with antibiotics (for example, penicillin) is the most effective treatment currently available in medicine. Silver has a more considerable number of advantages than its drawbacks (Firdhouse and Lalitha 2015).

Over the past two decades, the study of particles with sizes ranging from (1.0 - 100) nanometers that can penetrate the membrane of a living cell to explore and understand many of life's chemical reactions has gained prominence in molecular biology. In this field of biology, nanotechnology and its branch, known as nanobiology,haveprovided information due to their high ability to penetrate the membrane of a living cell. The ability to exert influence at the molecular and subcellular levels enables to facilitate the transfer of atoms and molecules through the microvasculature and into cancer cells. Thus, the transfer of nanoparticles containing drugs into cancer cells in the body results from their ability.

2.2.2 Synthesis of silver nanoparticles

Biosynthesis methods are cost-effective and environmentally friendly.Because capping or stabilizing is found in plant extracts or microbial cultures, making this approach safe and nontoxic. Interestingly, silver nanoparticles can be synthesized biologically (via biosynthesis) or through microbial fermentation as a by-productof this fermentation (Zuas *et al*. 2014).

2.2.3 Synthesis of silver nanoparticles by bacteria

Recently, numerous studies have been carried out to investigate the synthesis of silver nanoparticles using microorganisms as a potential Bio source. During the reduction of silver ions in bacteria, the time required to complete the reaction can range from 24 to 120 hours(Sabri *et al*. 2016).

The production of nanoparticles can occur extracellular or intracellular, depending on the bacterial species.After a certain amount of nanoparticle accumulation, bacteria can no longer survive.Then, nanoparticle accumulation can be toxic to the microorganisms involved(Duhan and Gahlawat 2014).

2.3 *Leishmania*Parasite

The*Leishmania* parasite is an important protozoan parasitic organism in medical research and treatment.They can live singly and in groups or colonies and carry out various metabolic activities and biological functions.Clear nuclei often characterize the parasites that cause Leishmaniasis.*Leishmaniae* are obligate intracellular parasites that cause Leishmaniasis.They live and reproduce within the macrophages of the vertebral host, where they were originally discovered.Many factors influence *Leishmania*diversity, including the parasite's behavior in humans, geographical distribution, endemicity, areas of presence, and the type of animal that stores the parasite and its transmission by different animals(Rassam and Al-Mudhaffar

1997).*Leishmania*parasite is transmitted to humans by sand fly vector(Akhoundi*et al*. 2016).

2.3.1 Morphology of parasite

It is found in the intestines of Phlebotomus and Lutzomyia vector (sand fly). In industrial culture media, the promastigote phase has a length of 15-20 micrometers and a width of 1.5-3.5 micrometers (Rittig and Bogdan 2000). The centrosome is at the front.In the vertebral host, it reproduces within the vertebral host's macrophages, making it immobile. In both forms, reproduction is accomplished through longitudinal fission, and the cytoplasm is clearly distinguished from the nucleus and contains mitochondria, vacuoles, and alkaline granules among other organelles and structures (Myles *et al*. 2007).

According to electron microscopy studies, the nucleus is located in the center of the site and directly in front of the movement's point of origin. The kinetoplast is surrounded by a nuclear envelope composed of two thin layers interspersed with minute holes and one thick layer with no holes. The Golgi apparatus is a small structure containing round and circular channels. It is located in the circumference of the flagellum pocket and is made up of two types of endoplasmic reticulum: the smooth endoplasmic reticulum, which has no ribosomes on its outer surfaces, and the rough endoplasmic reticulum, which has ribosomes on its outer surfaces (Al-Sabawi *et al*. 2021).

According to a recent study, cysts of varying sizes are abundant at the end of the *Leishmania* phase, which helps them survive within the phagocytic cells of the vertebral host (Figure 2.1)(Moradin and Descoteaux 2012).

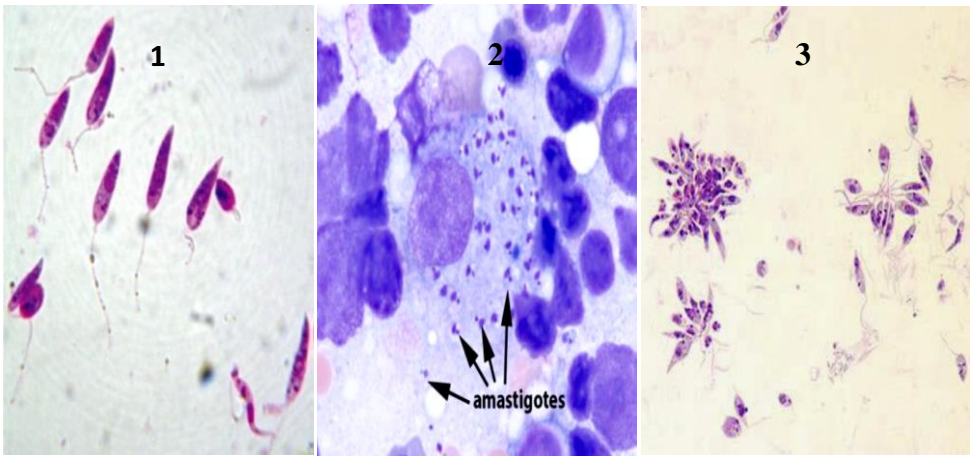

Figure 2.1 1- The flower shape of the Leishmania parasite after gathering; 2- Leishmaniasis within the phagocytic cell; 3- The gametophyte ofLeishmaniaparasite(Moradin and Descoteaux 2012)

2.3.2 Life cycle

A female sand fly bite infects a person with Leishmaniasis, one of the parasitic diseases of animal origin.Thesandflies are small insects. They fly during the evening, just slight above the ground, and make no sound. They generally live in hot and humid climates, in which their activities increase (Rebollar-Téllez *et al*. (2005), discovered that sand fly feeds on human or animal blood during summer (Rebollar-Téllez *et al*. 2005).The first step that occurs when it ingests the blood of an infected human or animal, such as (dogs or foxes), is the transformation of the lash less phase into the anterior flagella phase, which multiplies in the insect's stomach before reaching the anterior part of the middle intestine to settle in its saliva. When it bites a healthy human or animal, it injects the parasites causing diseaseinto his body (Figure 2.2)(Motazedian *et al*. 2006).

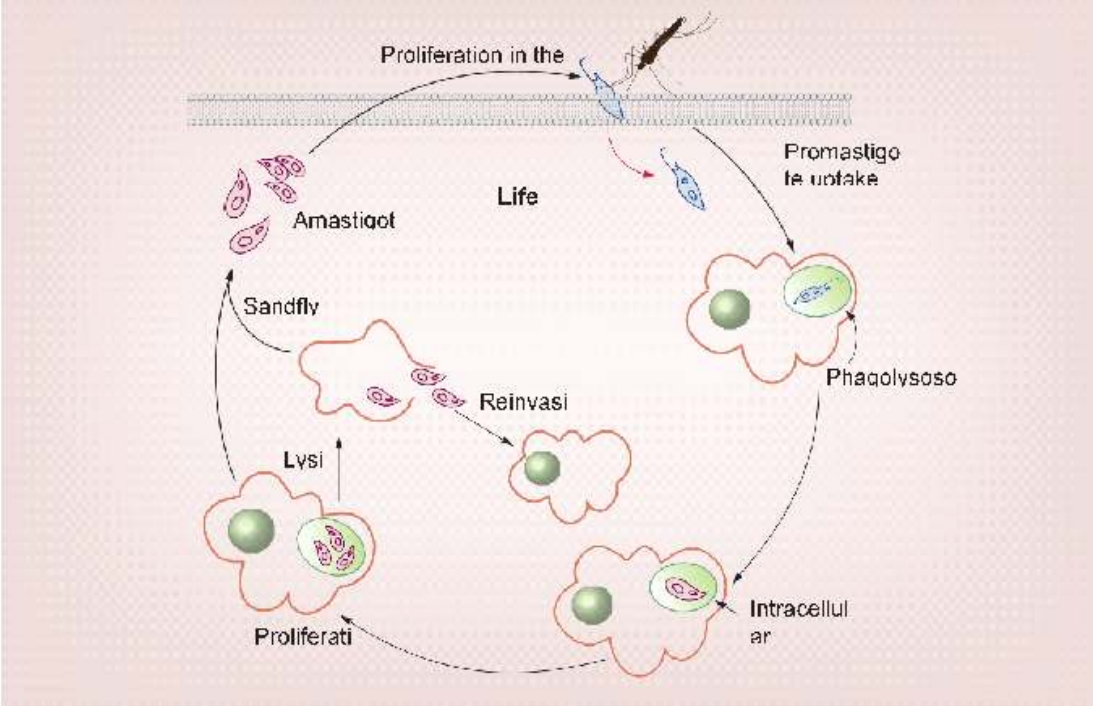

Figure 2.2 The life cycle of Leishmania parasite(Motazedian *et al*. 2006)

2.3.3 *Leishmania* taxonomy

Leishmaniasis was first classified based on ecological factors such as vectors, geographic distribution, tropism, antigenic characteristics, and clinical symptoms,.The biochemical and genetic study revealed that clinical and geographical criteria were lacking in many instances. Consequently, alternative criteria were utilized to identify *Leishmania*, such as polymorphism patterns revealed by kinetoplast DNA (kDNA) markers, proteins, or antigens.The parasite Leishmania belongs to the order Kinetoplastida because it possesses Kinetoplast, mitochondria, and the rest of the cytoplasmic organelles that belong to this order (Maurício 2018).

2.3.4 Pathogenesis of *leishmaniasis*

Leishmaniasis (CL) can cause severe infections and threaten human life, with occasional hosts and other animals, particularly (rodents and dogs), as in most parasitic

diseases. Pathogenesis in *Leishmania* species is complex, involving host immune responses and parasite virulence factors (Ridley 2004).

The most critical factors of virulence in Leishmaniasis are

Acid phosphatase enzymes (APE)

Acid phosphatase, also known as EC3.1.3.2, is a hydrolytic enzyme in many different microorganisms and is essential in developing cutaneous Leishmaniasis.It is believed that these enzymes originate in the Golgi apparatus and can be found in the endoplasmic reticulum, flagella receptors, and the surfaces of the outer membranes of various cells (Doyle and Dwyer 1993).

Cysteine protease (CP)

The Protease (also known as Leishmaniasis pathogenic enzyme or protective enzyme) is critical in the virulence of Leishmaniasis when the pH is at its optimal level.Its functions protect the parasite and degrade the host's immune system.When the macrophage successfully ingests the ammonia NH4 and the urea, the phagolysosomes' pH and the hydrolyzed proteins' pH are altered(Ferreira *et al*. 2003).

On the one hand, it is critical in binding the Leishmania parasite to the complement receptors for phagocytes CR1 and CR3, which are found on the surface of phagocytes(Huber *et al*. 1998).

Because the amastigote stage is more effective for Protease than the promastigote stage, the amastigote stage is credited with the role of virulence in the organism (Klein 2004, Chaudhuri and Chang 1988).

Lipophosphoglycan coat (LPG)

The parasite uses LPG to increase its chances of survival in its host and improve its ability to manipulate the host's immune response through various mechanisms.Macrophages can sustain their existence within them, but the parasites must avoid being killed by the phagocytic cell.Lipopolysaccharide plays a role in defending against the complement system, inhibiting both the initiation of an oxidative and inflammatory response and the ability of natural killer T cells to distinguish *Leishmania*-infected macrophages from non-infected macrophages(Wolday *et al*. 1999).

2.3.5 Epidemiology of leishmaniasis

The incubation period for various Leishmaniasis cases can last from a few days to several years. Still, it is typically between several weeks and six months in the case of cutaneous Leishmaniasis, for example.After the parasite has infiltrated and infected the vessels, primary skin lesions usually appear within several weeks after entering and infecting the vessels, with an average incubation period of 2 to 6 months.In any case, dogs are considered as a major reservoir for human infection in many endemic areas. On the other hand, humans are considered as the primary reservoir in other areas, which aids in spreading the disease (Faulde *et al*. 2008).

The disease is transmitted primarily by sandflies of the Phlebotomus (Old World) and Lutzomyia (New World).Both of them are found in the Old World.However, rare cases of Leishmaniasis have been reported through direct contact, exchange of blood, or other bodily fluids.These are the only known vectors capable of spreading.The epidemiology of Leishmaniasis is dependent on the parasite species, the environment, past and current human community exposure to the parasite, hostage, season, type, and structure of a building or house, geographical distribution(Yaseen *et al*. 2017).

2.3.6 Diagnosis of leishmaniasis

The diagnosis of cutaneous Leishmaniasis is based on pathology and clinical examination, and is confirmed by the presence of the parasite in the skin(Asgari *et al*. 2007).

Direct microscopic diagnosis

The sample is obtained from the lesion by isolating it or taking a biopsy to detect the parasite's amastigotes. Microscopically, it is stained with Giemsa stain or Romnowsky's stain, Haemotoxylin stain, Eosin stain, or Immunoperoxidase stain, which is more sensitive in the case of cutaneous Leishmaniasis, and then examined under a microscope.This examination is straightforward, inexpensive, and requires little knowledge (Motazedian *et al*. 2010).

The culture

It is possible to diagnose the parasiteby using culture media, such as the Novy-McNeal-Nicole NNN medium, initially described in 1908 by Novy and McNeilscientists and later modified by Nicole (Desjeux 2001).

At 26 degrees Celsius, promastigotes are grown on Novy, McNeal-Nicolle (NNN) media.This necessitates using a laboratory facility equipped with an incubator with cooling and sufficient experience and a long period during which samples are taken from blisters and biphasic media used in agriculture to produce large numbers of parasites. One thousand ten cells per milliliter of the medium, and rabbit blood are the primary components of this medium, preferred over other types.In this case, the implant's sensitivity is lower than the sensitivity of direct microscopy(Pourmohammadi*et al*. 2010).

Diagnostic tests using PCR technology with high sensitivity and specificity

PCR technique is a highly sensitive and specific method to diagnose leishmaniasis diseases using various biopsy materials, including ulcers, peripheral blood, lymph node biopsy, and other biopsy materials.It can also detect insect and animal parasites(Davami *et al*. 2011).

When DNA is extracted from cells or body fluids, the idea behind PCR technology is to amplify a few molecules to obtain more significant amounts of DNA, allowing us to perform the necessary analysis.It is essential to have certain materials available for this reproduction.These materials include:

1. Primers: Only a few nucleotides (18-20 oligonucleotides) contain a nitrogenous base capable of binding with the nitrogenous bases of the target nucleic acid, and they are located in a highly conserved region of the DNA with a distinctive and qualitatively unchanged arrangement of the nitrogenous bases.

2. Deoxynucleotide triphosphates (dNTPs) (dATP,dCTP,dGTP,dTTP).

3. The polymerase enzyme is resistant to high temperatures, the most important of which is Taq Polymerase, extracted from Thermos aquatic's, a bacterium living in hot springs.

4. Protective solutions (Buffers).

5. Suitable electrolytes are the most important magnesium ion Mg^{+2}, a complement factor for the polymerase enzyme (Bustin 2002).

Copy process:After placing the DNA to be copied with the primer and the polymerase enzyme and a group with the nucleic acids in a tube inside the thermostat, there are three separate stages for the transcription process:

1- Denaturation: The temperature is increased to 94°C to decode the original DNA.

2- Annealing: The temperature is reduced to a value between 55-60°C so that the primer sticks physically by hydrogen bonds with the original DNA.

3- Extension: Then the temperature is increased to 75°C for the polymerase to do its function in constructing the new DNA strand.Several cycles (30-40) in a programmed thermo cycler are required to complete the replication of a specific region of one of the two DNA strands in the presence of DNA primers(Fakhar *et al*. 2010).

2.4 *Leishmania* Genome

2.4.1 Nuclear genome

According to the projections, the core genome contains 911 RNA genes, 39 pseudo genes, and 8272 protein-coding genes (36% of which have a potential function).

The genome has a high G+C content (59.7 %).Genes in *Leishmania*are are frequently arranged in tandem arrays or have two or more copies distributed throughout the genome, depending on the species (Bard 1989).

According to the results, the rest comprises dispersed transposons, repetitive genes, and other basic sequences. So far, no introns have been detected in any of the *Leishmania* protein-encoding genes studied, making it easy to locate these genes in the parasite's genomic DNA. Even though interlocking distances vary amongst *Leishmania* species, initial comparisons with other genomes reveal that gene order is relatively constant among the 30 *Leishmania* species(Bussotti *et al*. 2018).

Although this is true, nucleic acid sequences exhibit a high degree of polymorphism (Ravel *et al*. 1999). Based on restriction patterns, Nevedetermined that nuclear DNA sequence diversity across the main *Leishmania* lineages varied from 13% to 25%. It was equivalent to the diversity found between animal species that disappeared from 10 - 80

million years ago. Despite this, nucleic acid sequences differ significantly (Beverley *et al*. 1987).

Several studies have shown that *Leishmania* chromosomes are mostly diploid (Beverley 1988). The parasite's ploidy level has been evaluated since gene amplification is a common approach utilized by *Leishmania*to withstand the impacts of cytotoxic or environmental stresses(Imamura *et al*. 2020).

2.4.2 Kinetoplast DNA

The Kinetoplastida's mitochondrial DNA (kDNA) contributes 10 – 20 % of the organism's total DNA (Simpson 1987). Homogeneous maxi circles (25–50 molecules of 20 kb) occur more often than heterogeneous minicircles (0.8 kb).

Although the maxi circle is the functional equivalent of mitochondrial DNA, it is involved in editing uracil residues into mRNA nucleotides(Mendoza-León *et al*. 1996).The cytochrome oxidase subunit III may be modified using guide RNAs (gRNAs) found in the minicircles (Sturm and Simpson 1990)..

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment and apparatus used in this study

In Table 3.1, Table 3.2, and Table 3.3, these three tables included some of vitro tools, instruments, chemical materials, and biological materials that used in the current investigation to arrive and get its goal.

Table 3.1 Chemical materials

	Materials	Company/ Origin
1	Agarose (cat. 8100.11)	Condalab / USA
2	Red Safe Staining Solution (cat. 21141)	Intron / Korea
3	6x Loading dye (cat.21161)	Intron / Korea
4	Ladder 100 bp & 1000 plus bp (cat. 24073 & 24075	Intron / Korea
5	Premix PCR (cat.25025).	Intron / Korea
6	Tbe buffer 10 X (cat. IBS.BT004).	Intron / Korea
7	Primers	Alpha DNA/Canada USA
8	Absolute methanol	Scharlau (Spain)
9	Quick-gDNA™ Blood MiniPrep (cat. D3024 & D3025)	Zymo Research/USA
10	Silver nitrate ($AgNO_3$)	Sigma Aldrich / USA
11	Potassium nitrate (KNO_3)	Adwic / EGYPT
12	Dimethyl sulfoxide (DMSO)	Sigma / USA
13	MTT	Sigma / USA
14	Sulfanilamide	Sigma / USA
15	N-(1-Naphthyl) ethylenediamine	Sigma / USA
16	Zinc powder	Sigma / USA

Table 3.2 Biological materials

	Materials	Company/ Origin
1	Agarose powder	Bio Basic/ (Canada)
2	Nutrient agar	Titan Biotech Ltd (India)
3	Nutrient broth	Alpha Chemika (India)
4	Roswell park medium (RPMI 1640)	Sigma (USA)

Table 3.3 Equipment and apparatus that used in this study

	Equipment and apparatus	Company / Origin
1	Aura ™ PCR Cabinet	Italy
2	Microspin12, High-Speed Mini-Centrifuge	Bio San / Germany
3	V-1 plus, Personal Vortex for tubes	Digsystem / Germany
4	Bio TDB-100, Dry Block Thermostat	Bio San / Germany
5	Micropipettes of variable sizes (Volume 0.5-10 uL) - (Volume 100-1000 uL)	Germany
6	Mini-Power Supply 300v, 2200v	China
7	Multi gene optimax gradient thermal cycler	Labnet / USA
8	Electrophoreses apparatus gel	CBS, Scientific / USA
9	Document system	Labnet / USA
10	UV transmission	Vilberlourmat / France
11	Microspin	Bio San / Latvia
12	Combi- Spin	Bio San / Latvia
13	Electrical sensitive Balance	Kernpfb / Germany
14	Incubation	Jrad / China
15	Microwave	Gosonic / China
16	Distilled Water	China
17	Laminar flow hood	K&K Scientific Supplier / Korea
18	Incubator shaker	LAB-Line / USA
19	Water bath	Labtech / Korea
20	Vortex	Dubuque / USA
21	Centrifuge	Hereaus / Germany
22	Eppendorftubes	China
23	Light Microscope	Olympus / Japan
24	96 – well Microtiter Plate	Flow Lab., Irvine / U.K
25	Refrigerator	Indesit / Turkey
26	Ph-Meter	Radiometer / Denmark
27	ELISA	QuikFit / Germany
28	Oven	Memaret / Germany
29	Autoclave	Selecta / Spain
30	Cylinder (25ml, 250ml,500ml)	Memmert / China
31	Conical flask (250ml, 500ml)	Memmert /China
32	UV–visible spectrophotometer	Shimadzu / India
33	(FTIR)	Japan
34	(TEM)	Broker/Germany
35	(EDX)	Broker/Germany
36	Plain tube	Jordan
37	Bunsen burner	Jenway / Germany
38	Wire Loop	John Bolten /England
39	Medical Cloves	Omega / China
40	Marking Pen	Doms / India

3.2 The Methods

3.2.1 Procedures and plan

The main steps of the research plan are illustrated in Figure 3.1.

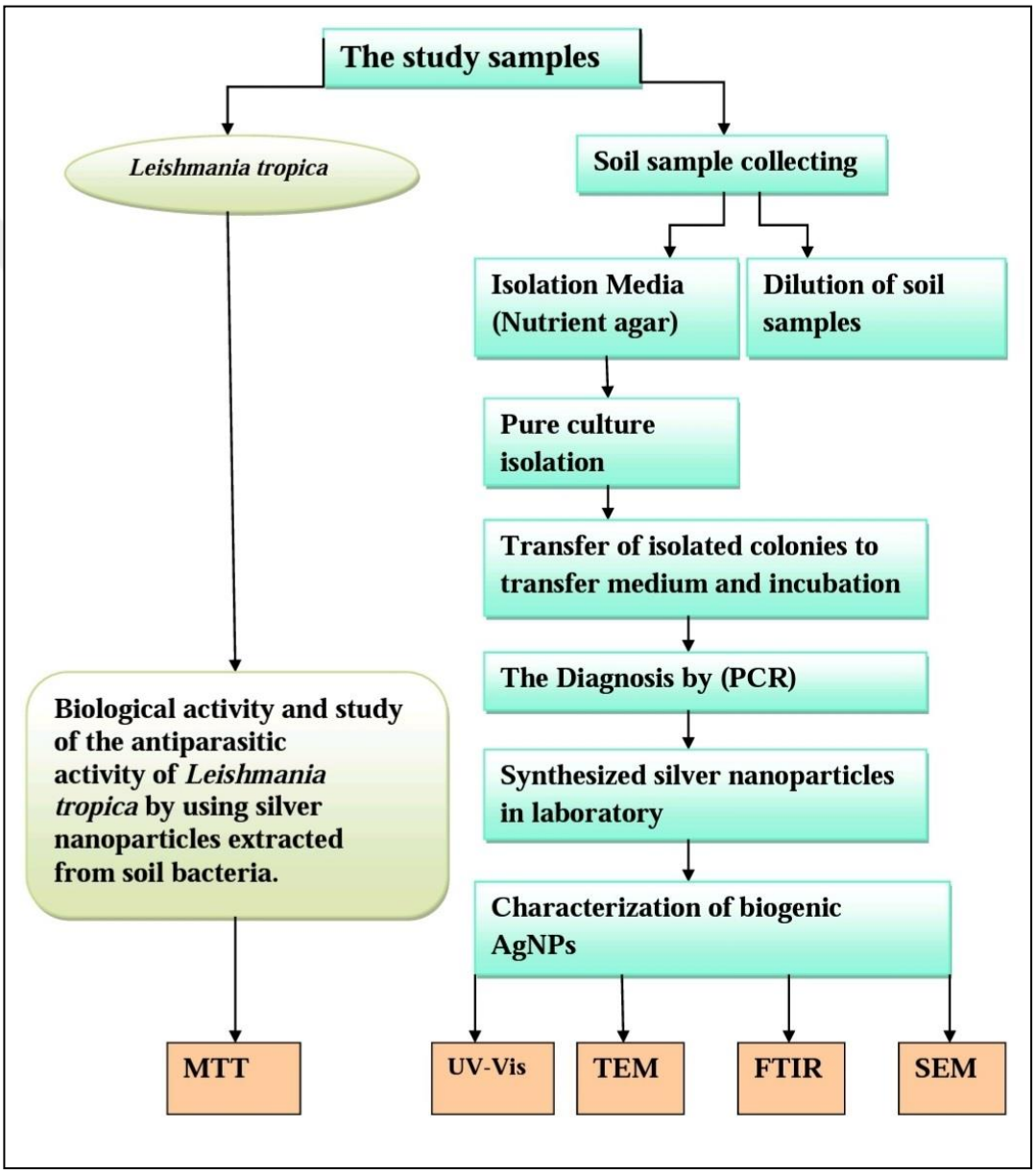

Figure 3.1 The main steps of the research plan

3.2.2 Soil sample collecting, isolation, and dilution

In this study, soil samples was gathered by digging from the ground's surface to a depth of 11mm using a trowelin several locations, including sugar cane fields and gardens. The samples were put in sterile tiny plastic bags with labels showing the date and place of collection.

In this study, bacteria were isolated using Nutrient agar. Nutrient agar medium was made, and the components were placed in the flask, which was then covered with cotton and heated on a heater to dissolve them. After it was cooled down, the pH was checked and adjusted to 7 - 7.2 before it was sterilized in an autoclave at 121°C for fifteen minutes. The medium was then allowed to cool to roughly 50°C and then poured into Disposable Petri dishes.

The numbers 1, 2, 3, and 4 were written on four sterile test tubes filled with 9 mL of sterile distilled water. In the test tube 1, one gram of dry soil sample was put and thoroughly mixed. Using micropipette, one ml of soil solution was transferred from the tube 1 to the tube 2 and carefully mixed. This repeated dilution was completed until the tube 4 was reached. The tube 4 was add with the 1ml solution to obtain the concentrations (10 - 1, 10 - 2, 10 - 3, 10 - 4).

3.2.3 Preparation of the culture medium and dilution plating

The culture medium (Nutrient agar) was prepared according to the manufacturer's instructions (Titan Biotech Ltd /India), for dissolving 28.0 g in 1000 mL distilled water. It was gently boiled and constantly stirred until the medium was entirely dissolved. It was sterilized in an autoclave at 15 bars of pressure (121°C) for 15 minutes. Last, it was left to cool down to 45-50°C, and then the medium was dispensed.

A total of four Petri dishes containing isolation media were labeled 1, 2, 3, and 4 to correspond to each dilution test. 0.1 mL of the solution was transferred to the labeled

Petri plates starting with the tube 4. Each soil solution was distributed on the agar medium immediately after it was transferred using a sterilized Wire Loop. The plates were then incubated for one day at 37°C.

3.2.4 Identification of the isolates

Identification of the gene:

Phylogenetic relationships of bacteria and all life forms may be identified by comparing a stable component of the genetic code. In bacteria, this genomic region is defined by the 5S, 16S (also known as the small subunit), and *23SrRNA* genes, as well as the gaps between them. *16SrDNA* is a colloquial term for the *16SrRNA* gene. The ASM's current guideline refers to it as the "*16SrRNA* gene." The *16SrRNA* gene may be compared with other bacteria, archaeobacteria, and eukaryotes'*18SrRNA* gene.

The *16SrRNA* gene sequence is 1550bp in length and is composed of variable and conserved regions. The gene is big enough to provide distinguishing and statistically significant measurements and exhibits a sufficient inter-specific variation.

Generally, universal primers supplement the conserved regionsat the start of the gene, at the 540bp region, or after the whole sequence (about the 1550bp region).

It is considered whether or not it is required to sequence the complete 1500bp length or the often reported shorter sequences may provide equivalent information.

It is sometimes necessary to sequence the whole 1500-bp region in order to differentiate specific taxa or strains.

When describing a new species, it is also desirable and often necessary to sequence the 1.500bp sequence (Clarridge III 2004). Because the *16SrRNA* gene is prevalent in all bacteria, relationships between species may be examined. Generally, the comparison of

16SrRNA gene sequences allows separating the genera across all major phyla of bacteria and classifying strains at several levels, including species and subspecies.

The only real-time *16SrRNA* gene sequencing is ineffective when more than one well-known species has a sequence that is identical or substantially similar.

*16SrRNA*gene sequencing:

Several methods were used in the past to determine DNA sequence. Genomic DNA was isolated from isolates using a universal primer. The primer sequence are illustrated in Table 3.4.

Table 3.4 Primer kit (Miyoshi *et al*. 2005)

Primer	Sequence (5´- 3´)	Length	Amplicon Size
27F	AGAGTTTGATCCTGGCTCAG	20 bp	1500bp
1492R	GGTTACCTTGTTACGACTT	19bp	

DNA extraction

Presto' mini g DNA bacteria kit quick protocol TM, catalogue number DO GBB004, GBB100 / 101, GBB300 / 301 was the procedure of DNA extraction in this study.

The PCR conditions

Following DNA extraction, the DNA must be amplified using PCR (polymerase chain reaction). The PCR procedures were as follows:

A one-minute denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. The amplified PCR product was then electrophoresed on agarose gel (Pharm 2008).

Electrophoresis on agarose gels

The electrophoresis tank was filled with TBE 1X buffer, and the tray holding the previously used agarose was submerged. Each well had 5 uL of PCR product.

Then, a DNA ladder with a standard molecular weight (5 uL) was added to the first well. The gel was inspected and photographed using a gel documentation device after 80-minute electrophoresis at 80 volts (Green and Sambrook 2019).The product which was photographed was written in the results chapter.

Genotypic identification on genbank

The PCR product was collected from the gel and transmitted to the biotechnology laboratory for sequencing at the national instrumentation center for environmental management (nicem) online (http://nicem.snu.ac.kr/main/?en skin=index.html) using an Applied Bio systems DNA Sequencer 3730XL. Homology searches were undertaken using the basic local alignment search tool (BLAST) software, which is freely accessible online at the NCBI website (Hassan 2018**)**.

3.2.5 Screening of bacteria to determine nitrate reductase production

The nitrate reduction test determines the synthesis of the nitrate reductase, which results in nitrate reduction (NO_3). This experiment was carried out to determine which nitrate reductase bacteria can convert nitrate to nitrite in producing silver nanoparticles, depending on the method (Bhusal and Muriana 2021).

3.2.6 Synthesized silver nanoparticles in a laboratory

Biosynthesis of Silver nanoparticles from the isolation strains of bacteria

Bacterial strains isolated, purified, and genotypically identified were cultivated in nutrient broth (pH = 7) at 37 °C for 7 days by being continuously shaken in a rotary shaker (150 rpm).

After incubation, biomass was filtered using filter paper (No. 1) to remove it from the supernatant, and the medium was centrifuged to get the supernatant(Singh *et al*. 2018). To produce AgNPs, two solutions were prepared: the first one was prepared by mixing 200 mL of supernatant with 200 mL of $AgNO_3$ (v/v) (2 mM).

A second reaction mixture was prepared without $AgNO_3$ and used as a control. The interaction occurred at a pH of 7.0. The interacting solutions were kept in the rotary shaker (150 rpm) at 37°C (to prevent photochemical reversal during the experiment) in the dark for 7 days. The emergence of a significant change in the culture fluid was the first indication for development of Bio-AgNPs (Dayma *et al*. 2019).

Silver nanoparticles are synthesized extracellular by utilizing the supernatant. Simultaneously about 2 g of wet biomass was suspended in 100 ml of 2 mM aqueous AgNO3 solution with collecting bacterial biomass for intracellular synthesis(Das *et al*. 2014).

The mixture was separated at 8000 rpm for 20 minutes, the supernatant was replenished with DDW, and the pellet was dried at 45 °C for 30 hours. The powder was preserved in vials for subsequent examination (Aldujaili*et al*. 2020) so that the silver nanoparticles were obtained.

Characterization of biogenic AgNPs

Physical and biological characterizations are the most common methods for determining the properties of nanoparticles.

Physical characterization of biogenic AgNPs:

Size and shape are two of the most significant properties studied in nanomaterial characterization. Volume distribution, aggregation degree, surface charge, surface area, and, to a lesser extent, surface chemistry may all be quantified. Particle size, volume distribution, and organic bonding on the particle's surface may all impact other properties and applications. Furthermore, their crystal structure and chemical composition are thoroughly analyzed as a preparatory step after nanoparticle production(Verma and Maheshwari 2019).

A- UV- Spectroscopy

A UV–visible spectral scan at 200-800 nm was used to ensure the production of silver nanoparticles. Both treated and untreated solutions were centrifuged for 5min at 2000 rpm. The untreated supernatant was set as a reference control while treated supernatants were used to monitor their UV-Visible absorbance Spectra between 200-800 nm wavelengths. Silver nanoparticles have a broad UV visible spectrum indicating their presence (Soltaninezhad *et al*. 2015).

B- TEM examination

Micrographs taken using Transmission Electron Microscopy (TEM) were utilized to identify the formation type (shape) and size of the generated silver nanoparticles (Williams and Carter 1996).

Biological characterization of biogenic AgNPs:

The impact of Ag-NPs on *L. tropica* metabolic activity (viability) at different concentrations (12.5, 25, 50, 100, 200, 400 µg /ML) was assessed microscopically and quantitatively using the MTT technique in the dark.

A- The MTT technique

The MTT cell viability was conducted on 96-well plates. *L. tropica* was seeded at $1 \times_{10}^{4}$ parasites/well., Parasites were treated with the tested compound at final concentration (400) µg/mL 24h later or after a confluent monolayer was achieved. Parasite viability was measured after 24 hours of treatment by removing the medium, add 10 µL of MMT (0.45 mg/mL solution) then incubate the parasites at 27 °C for two hours, solubilized the crystals by adding 100 µL of DMSO and then incubate at 27 °C for five minutes. (Al-Shammari *et al*. 2019). The absorbency was determined on a microplate reader at 629 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of parasite growth (the percentage of cytotoxicity) was calculated as the following Equation 3.1.

$$Proliferation\ rate\ as\ (PR)\ =\ B/A * 100 \quad (3.1)$$

Where A is the mean optical density of untreated wells and B is the optical density of treated wells and IR= 100- PR (Freshney 2015).

Preparation of AgNPs solution:

i- The suspension was prepared by adding 400 µg/mL of AgNPs to 1 mL of RPMI-1640.

ii- Drops of DMSO were added to the suspension to dissolve the Ag-NPs.

iii- Half dilutions were prepared by taking 0.5 mL of suspension, the prepared tube and supplementing it to 1 mL with RPMI 1640. In the same way, 5 glass tubes containing 5 concentrations of AgNPs were prepared, thus 6 tubes containing 6 concentrations of Ag-NPs were obtained as follows: 12.5, 25, 50, 100, 200, and 400 µg/mL.

Collection and cultivation of leishmania parasites:

In this study a standardized strain of the *Leishmania* parasite was received from the Department of Biomedical and Molecular Technologies at the University of Baghdad. *L. tropica* was grown and replicated in nutrient RPMI 1640 medium supplemented with penicillin (100 unit mL^{-1}), streptomycin (100 mg/mL^{-1}), and fetal calf serum (10 percent v/v) and incubated at 27 °C.

Maintaining and growing of leishmania tropica parasites:

This method was followed (Freshney 2015).

- Parasites were placed in tubes containing RBMI-1640 culture medium and 10% bovine calf serum.

- The solution containing Ag-NPs, as well as the culture medium, was incubated in an incubator at 27°C for twenty-four hours, and after a day of incubation, and when it was confirmed that there was growth in the cell farm and it was free of contamination, secondary farms were conducted.

- Parasites were examined with a light microscope on a 40X lens to ensure that they were alive, free of contamination, and growing to the required number.

- The cells were transferred to the growth cabin, and the used culture medium was disposed of.

- The cells were rinsed with PBS solution and discarded, and the procedure was done twice for a total of 15 minutes.

- The number of cells was examined by taking a certain volume of the suspension containing the parasites, adding to it into the same volume of trypan Blue dye to

find out the number of cells and their vitality percentage using a Hemocytometer slide according to the following Equation 3.2.

$$C = N * 10^4 * F/mL \quad (3.2)$$

C = Number of parasites in one mL of solution

F = Dilution factor

N = The number of parasites in the slide$\mathbf{10^4}$**=** Slide Dimension

The percentage of parasite vitality in the sample was calculated using a Hemocytometer chip according to the following Equation 3.3.

$$\text{Percentage of live cells vitality} = \frac{\text{live cells}}{\text{dead cells}} \times 100\ \% \quad (3.3)$$

Anti-Leishmania study of the synthesized AgNPs in vitro:

- MTT assay for parasites' vitality

The MTT test is a simple, quick, and dependable method for measuring the proportion of viable parasites (Morgan 1998, Meerloo *et al*. 2011). The MTT test was carried out following the technique of (Mosmann 1983). Each sample was tested in 18 wells of a 96-well microplate.100 µL of the solution containing 1×10^4 parasites plus 10 µL of MTT stock solution (0.45 mg/mL) was placed into each well. They were measured using ELISA at 620 nm after four hours of incubation at 27°C.

- Cytotoxicity assays, according to the method (Mahmoudvand *et al.* 2016)

1- The parasites of *L. tropica* were prepared according to the condition of parasite permanence and growth (1×10^4parasites/mL) and then the solution was placed in a 96-well plate with a flat bottom. The plates were covered with sterile parafilm gently and then stirred. They were incubated at 27°C for 24 hours before removing the medium.

2- After incubation, 100 µL of RBMI-1640 medium containing parasites was added to each well.

3- The prepared concentrations of silver nanoparticles (Ag-NPs) as 125, 25, 50, 100, 200, and 400 µL were added.

4- The plate was incubated at 27°C for 24 hours.

5- Each plate was filled with 10 µL of 0.45 mg/ml MTT solution.

6- The plate was incubated at 27°C for 4 hours.

7- Dissolve the crystals, by adding 100 µL of solubilization solution to each plate and incubated for 5 minutes then measured with ELISA equipment.

8- Set the wavelength at 620 nm, then read the optical density and calculate parasite vitality, statistical analysis of optical density readings was made according to the following Equation 3.4.

$$\text{Viability (\%)} = \frac{\text{the optical density of the sample}}{\text{the optical density of the control}} \times 100\% \quad (3.4)$$

3.3 Molecular Study of *Leishmania*Parasite

3.3.1 DNA extraction

Below is the DNA purification of 10 µL of the culture medium used for the growth of the parasite (RPMI 1640) (volumes can be adjusted up to 200 µL (maximum) as per your requirements).

1- Add 400 µL of Genomic Lysis Buffer to 10 µLof the culture medium utilized for the parasite's growth (RPMI 1640) (4:1). Vortex the mixture for 4 - 6 seconds, then let it for 5 - 10 minutes at room temperature then add 200 µL of Genomic Lysis Buffer. Add 800 µL of Genomic Lysis Buffer to 200 µL of blood.

2- The mixture was transported to a collection tube containing a Zymo-Spin IIC^{TM}Column2. Centrifuge the mixture for 1 minute at 10.000 x g. then remove the collection tube from the flow.

3- The mixture was transferred to a new collection tube, add 200 µL of DNA pre-wash buffer to the spin column. Centrifuge for 1 minute at 10,000 g.

4- Add 500 µL of DNA wash buffer to the spin column. Centrifuge for one minute at 10.000 g.

5- The mixture was transferred to a clean microcentrifuge tube. Add the DNA elution buffer to the spin column3 then incubate for 5 minutes at room temperature centrifuge for 30 seconds to elute the DNA. The eluted DNA may be utilized immediately for molecular applications or kept at-20°C for later use. Table 3.5 shows the components of the maxime PCR PreMix kit (i-Taq).

Table 3.5 The components of the maxime PCR PreMix kit (i-Taq)

Material	Volume
i-Taq DNA Polymerase	5 U/µL
DNTPs	2.5 mM
Reaction buffer (10X)	1 X
Gel loading buffer	1 X

3.3.2 Agarose gel electrophoresis of DNA

Electrophoresis was performed on the PCR product to determine DNA pieces after the extraction process or to detect the result of the PCR interaction in the presence of the standard DNA to differentiate the bundle size of the result of the PCR interaction on the Agarose gel.

Preparation of the agarose gel:

Sambrook *et al*. (1989), the 1.5 % condensation agarose gel was created by melting 1.5 g of agarose in 100 mL of previously prepared TBE buffer. Agarose was boiled and then allowed to cool down at 45-50 ºC. After attaching the comb to produce holes containing the samples, the gel was poured into the pour plate where the plate of agarose support was made. The gel was poured carefully to avoid air bubbles and allowed to cool for 30 minutes. The comb was carefully withdrawn from the firm agarose. The plate was attached to its stand in the electrophoresis horizontal unit, represented by the tank used in the electrophoresis. The tank was filled with TBE buffer, which was applied to the gel surface.

Preparation of sample

After mixing, 3 µL of the processor loading buffer (Intron/ Korea) was coupled with 5 µL of the supposed DNA to be electrophoresed (loading dye); the loading dye was in the pores of the gel. An electric current of 7 vc2 was applied for 1-2 hours or until the tincture reached the opposite side of the gel. After being put in a pool containing 3 µL

of red safe nucleic acid staining solution and 500 mL of distilled water, the gel was inspected with a UV light at 336 nm, and the process steps are shown in the Figure 3.2.

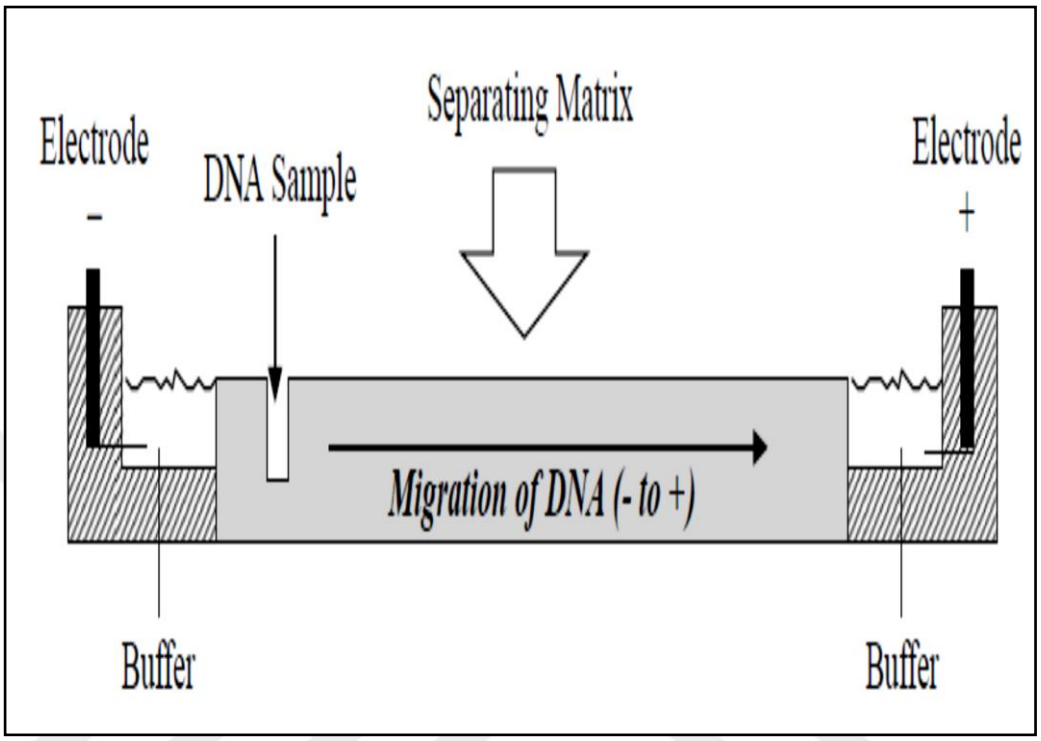

Figure 3.2 The common setup for DNA agarose gel electrophoresis. The top panel depicts a cross-section of the gel electrophoresis device

Red safe nucleic acid staining solution

Red safe nucleic acid staining solution (20.000x) is a unique and safe nucleic acid stain that may be used instead of the standard ethidium bromide (EtBr) stain to detect nucleic acid in agarose gels. It emits green fluorescence when linked to DNA or RNA. This new dye has two fluorescence excitation maxima linked to nucleic acid, one at 309nm and the other at 419nm. There is also one visible excitation at 514nm. The fluorescence emission from Red Safe bound to DNA is centered at 537nm.

The sensitivity of red safe nucleic acid staining solution (20.000x) is comparable to that of EtBr. Red safe nucleic acid staining solution (20.000x) staining is equivalent to EtBr staining. Red safe nucleic acid staining solution (20.000x) produces much fewer

mutations than EtBr, a known potent mutagen, in the Ames test. Consequently, red safe nucleic acid staining solution (20,000x) is superior to EtBr for recognizing nucleic acid in agarose gels (Cat. No. 21141).

3.3.3 Detection of *Leishmania tropica* by polymerase chain reaction (PCR)

The primers used in the interaction

The primers were lyophilized, diluted in free distilled water at a final concentration of 100 pmol/µL as a stock solution, and stored at -20 to create a stock solution of 10 pmol/µL as a working primer (see Table 3.6). IDT tested 10 µL of the stock solution in 90 µL of free distilled water to obtain a final volume of 100 µL (Integrated DNA Technologies company, Canada).

Table 3.6 The specific primer of gene LITSR/L5.8S (El Tai *et al.* 2001)

Primer	Sequence	TM (°C)	GC (%)	Product size
Forward	5′-CTGGATCATTTTCCGATG-3′	49.73	52. 63	300 - 350 Base pair
Reverse	5′-TGATACCACTTATCGCACTT -3′	50.68	47.36	

Protocol of PCR

a. The pre-mix Master Mix (cat. 25025) was thawed at room temperature. The master mix was vortexes then shaken for a short time.

b. A reaction mix was prepared for each sample by mixing the components shown in the Table 3.7.

c. The mixture was then put in a microcentrifuge and spinner for better mixing.

d. After mixing all the components, the tubes were transferred to the thermo cycler preset based on the program shown in Table 3.8.

e. The product of the polymerase chain reaction (PCR) and electrophoresis, 3L of DNA ladder and 5 L of PCR products were loaded into agarose gel at 2% (1x TBE Buffer) at 5 V/cm 2 for 1 hour. The gel was stained with Red Safe Nucleic Acid staining solution. Bundles were seen by UV transmission (302 nm) and photographed using the Document system (Beiranvand *et al*. 2013) shown in the Table 3.9.

Table 3.7 Mixture of the specific interaction for diagnosis of L5.8S/ LITSR gene

Components	Concentration
Taq PCR PreMix	5 µL
Forward primer	10 pmol/µL (1 µL)
Reverse primer	10 pmol/µL (1 µL)
DNA	1.5 µL
Distill water	16.5 µL
Final volume	25 µL

Table 3.8 Optimum L5.8S/LITSR gene detection methodology

No	Phase	TM (°C)	Time	NO. of cycle
1	Initial Denaturation	94°C	5 min.	1 cycle
2	Denaturation -2	94°C	30sec.	30 cycles
3	Annealing	53°C	30 sec.	
4	Extension-1	72°C	30 sec.	
5	Extension -2	72°C	10 min.	1 cycle

Table 3.9 The specific primer of gene mkDNA (Pereira *et al.* 2014, Disch *et al.* 2003)

Prımer	Sequence	TM (°C)	GC (%)	Productsıze
Forward	5′- GGGKAGGGGCGTTCTSCGAA-3′	58.84	47.61	120 Base pair
Reverse	5′-SSSWCTATWTTACACCAACCCC -3′	49.17	52.38	

PCR Technique

1. The pre-mix Master Mix (cat. 25025) was thawed at room temperature. The master mix was vortexes before being spun in the micro centrifuge for a short while.

2. For each sample, a reaction mix was created by mixing the components shown in the Table 3.10.

3. The mixture was then placed in a microcentrifuge and spinner to ensure a proper mixing.

4. After thoroughly mixing all the components, the tubes were transported to the thermo cycler, which was pre-set based the protocol provided in Table 3.11.

5. the product of the polymerase chain reaction (PCR) and electrophoresis , 3L of DNA ladder and 5L of PCR products were loaded into agarose gel at 2% (1x TBE Buffer) at 5 V/cm 2 for 1 hour. The gel was stained with red safe nucleic acid staining solution .Bundles were seen by UV transmission (302 nm) and photographed using the Document system (Beiranvand *et al*. 2013).

Table 3.10 Mixture of the specific interaction for diagnosing mkDNA gene

Components	**Concentration**
Taq PCR PreMix	5 µL
Forward primer	10 pmol/µL (1 µL)
Reverse primer	10 pmol/µL (1 µL)
DNA	1.5 µL
Distill water	16.5 µL
Final volume	25 µL

Table 3.5 Optimum kDNA gene detection methodology

No	**Phase**	**TM (°C)**	**Time**	**NO. of cycle**
1	Initial Denaturation	94°C	5 min.	1 cycle
2	Denaturation -2	94°C	30sec.	30 cycles
3	Annealing	53°C	30 sec.	
4	Extension-1	72°C	30 sec.	
5	Extension -2	72°C	10 min.	5 cycles

DNA sequencing

The polymerase chain reaction products for 2 samplesof *L.tropica* were sent to Macrogen Company for sequencing in order to ensure the diagnosis results.

The obtained sequences were aligned using NCBI software with normal NCBI GenBank and then examined. Furthermore, the genes recorded in GenBank are mentioned in the results chapter.

4. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Isolated bacteria from soil

Only three types of bacteria were used after the plates were inoculated with soil suspension that had been incubated for 24 h using serial dilution technique. Then pure bacteria cultures were obtained on nutrient agar after 24 hours of incubation at 37°C. Data were collected from all soil sources, and the selected colonies were distinguished based on their differences in colony shape and color diversity.

Thecomposition ranged from waxy to gelatinous, as the environmental bacteria *Pseudomonas aeruginosa* strain ALK320, *Bacillus cereus* strain HNB5 and *Corynebacteriumstriatum* strain 1910ICU141 were used as a source for the biosynthesis of silver nanoparticles after identification by polymerase chain reaction (PCR) technique.The results showed that the three bacterial species selected in this study were consistent with (Silambarasan and Abraham 2012, Kumar and Mamidyala 2011).

4.1.2 Molecular phylogenetic analysis and characterization

DNA extraction

Extracting Genomic DNA from the isolated bacteria was done using this method Presto' Mini g DNA Bacteria Kit Quick Protocol TM, Catalogue Number DO GBB004, GBB100 / 101, GBB300 / 301.

DNA amplification by PCR

The *16SrDNA* gene was partially amplified after extracting genomic DNA from bacteria using *16S rRNA* gene universal primers.

Agarose gel electrophoresis

It was used as anagarose gel electrophoresis and documented.The results from 16sDNA clear bands were at 1500bp in size. Detected the chosen isolates generated amplicons with an electrophoresed at 1% agarose gel at 80 volts for 80 minutes using a UV gel viewer which is shown in Figure 4.1.

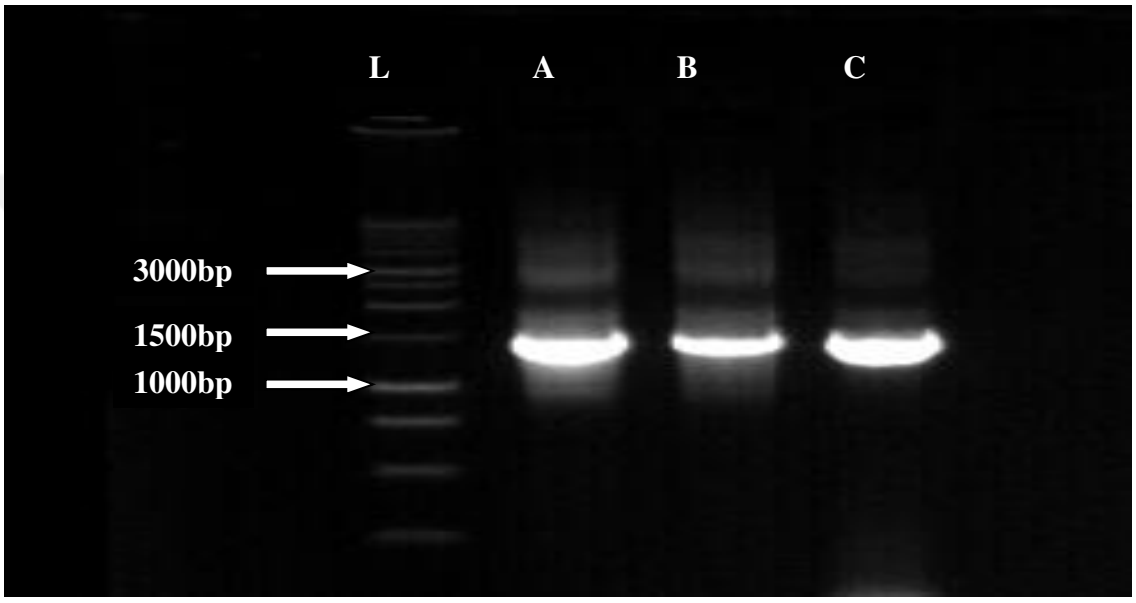

Figure 4.1 Genomic DNA extracted from three bacterial isolates. Lane (L) represents the molecular ladder (100)bp, and Lane (A, B, and C) represents the positive PCR product size (1500bp) of universal primer. (A) *Pseudomonas aeruginosa* strain ALK320. (B) *Bacillus cereus*

Sequencing of the *16SrDNA* gene

16SrRNA gene sequencing showed that the isolated bacterial species were similar to bacteria in GenBank (Table 4.1), and the obtained sequences of this study were submitted to the NCBI database, and accession numbers were received;ON340727, ON340740 and ON340743.

Table 4.1 Recorded bacterial strains in GenBank

Accession NO. of new strain	Sequence identity with reference strain (%)	Closed species	Accession number	Isolates code
ON340727	89%	*Pseudomonas aeruginosa*	KC456535.1	S1
ON340740	92%	*Bacillus cereus*	MW485810.1	S2
ON340743	99%	*Corynebacterium striatum*	MT225764.1	S3

4.1.3 Production of extracellular AgNPs in laboratory

After adding silver nitrate ($AgNO_3$, 1mM) as a substrate for the cell-free supernatant, the strain used for the biosynthesis of AgNPs showed the ability to biosynthesize extracellular under previous optimal conditions.

After incubation in a shaking incubator at 150 rpm, the color of the reaction mixture changed from light yellow to reddish-brown, indicating that the bacteria were bio actively synthesizing AgNPs, as shown in Figure 4.2.

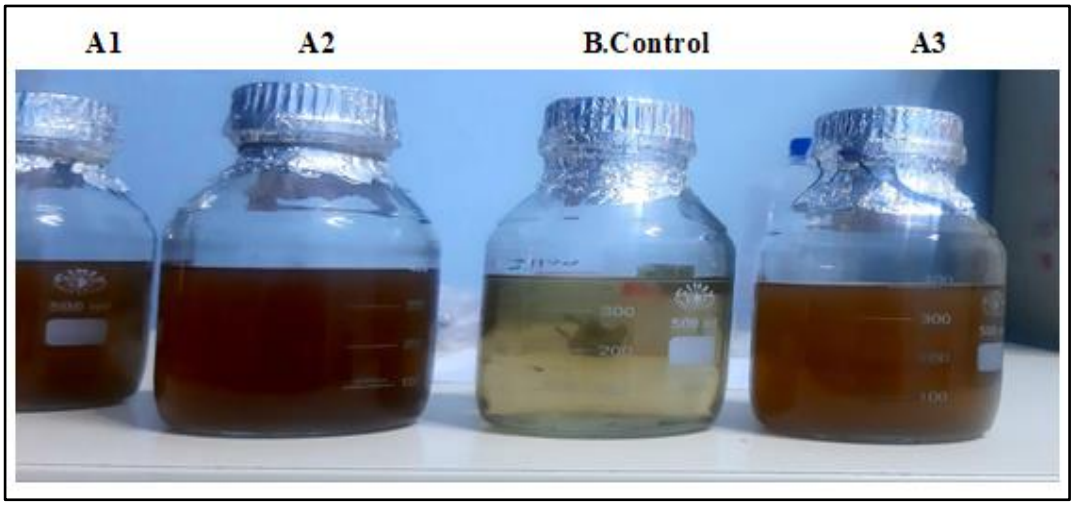

Figure 4.2 Extracellular synthesis and color change of supernatant, A1-A2-A3 with 1mm $AgNO_3$, after 72 h.B. without 1mm $AgNO_3$ (control) after 72 h

This means that the isolated bacteria had the enzymes (nitrate reductase) produced by cell activities that targeted metal ions from their environment convert (Figure 4.3).

Theincrease in the kinetics of the deposition of the silver atoms after Ag^{+} convert to Ag^{0} resulting in the extracellular creation of AgNPs (Elbeshehy *et al.* 2015). In this experiment, the biomass did not produce AgNPSfor intracellular biosynthesis, as shown in theFigure 4.3.

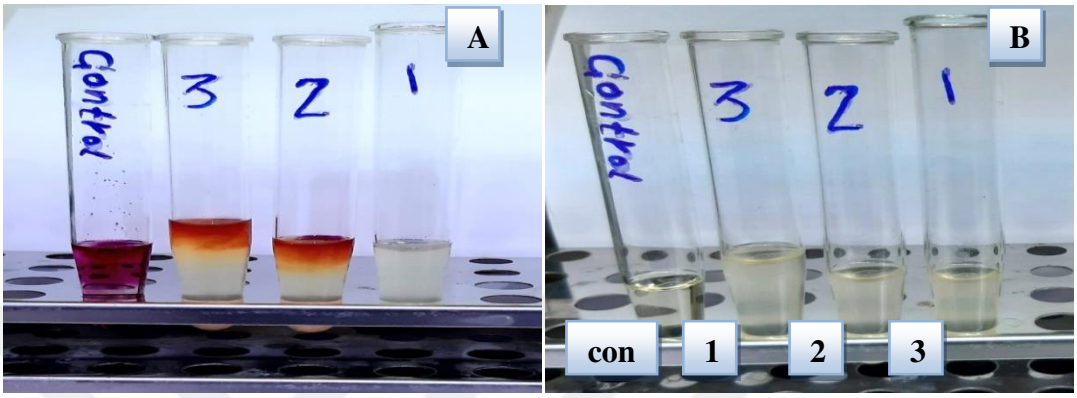

Figure 4.3 (A) Before adding reagents, (B) After adding reagents, the three species of isolated bacteria had the enzymes (nitrate reductase), (1) *Pseudomonas aeruginosa* strain ALK320, (2) Bacillus cereus strain HNB_5, (3) *Corynebacterium striatum* strain 1910ICU141

Afterward, the nanoparticle solutions were put in Pyrex glass plates and drying at 60°C for 30 hours. Then scratched the nanoparticles and retained the powder in plates for further analysis.

4.1.4 Characterization of silver nanoparticles

Physical characterization of AgNPs:

1- UV-Vis spectroscopy

The intense color of the distributed silver nanoparticles was due to the absorption of the surface Plasmon resonance spectrum (SPR) of silver nanoparticles.

Metallic bio nanoparticles, as a result, had a distinct optical absorption spectrum in the UV visible region and exhibited a distinct optical absorption spectrum in the UV-visible range. AgNPs had a significant UV–visible spectrum, as shown in Figure 4.4.

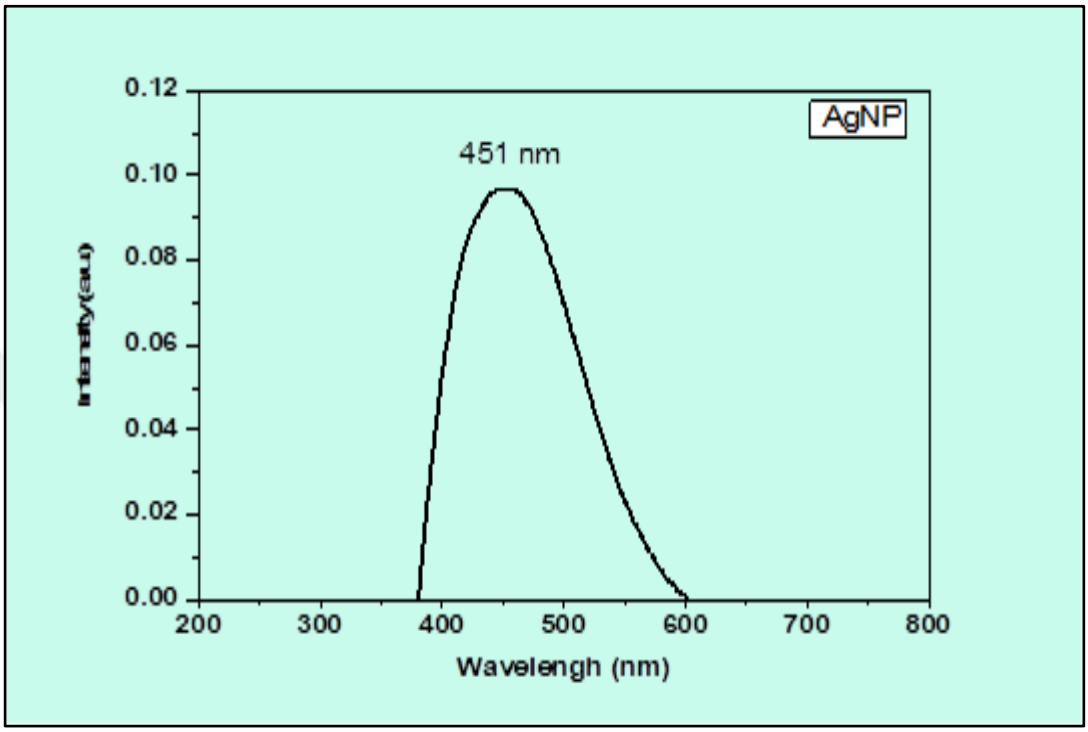

Figure 4.4 UV spectroscopy for the synthesized silver nanoparticles

TEM analysis

The size and morphology of particles were assessed using the TEM (Figure 4.5). The TEM image shows silver particles with an average diameter of about 20- in all samples. The present data agree with the study by (Singh 2015).

TEM images show that the AgNPs had a narrow size distribution, which corresponded to the shape of UV-visible spectra. Some agglomerates can be observed. The TEM micrograph in Figure 4.5 indicated mono-dispersed silver nanoparticles with spherical shapes. The micrograph of synthesized nanoparticles at 20 nm magnification showed an average size ranging between 12.687 and16.289 nm and was spherical.

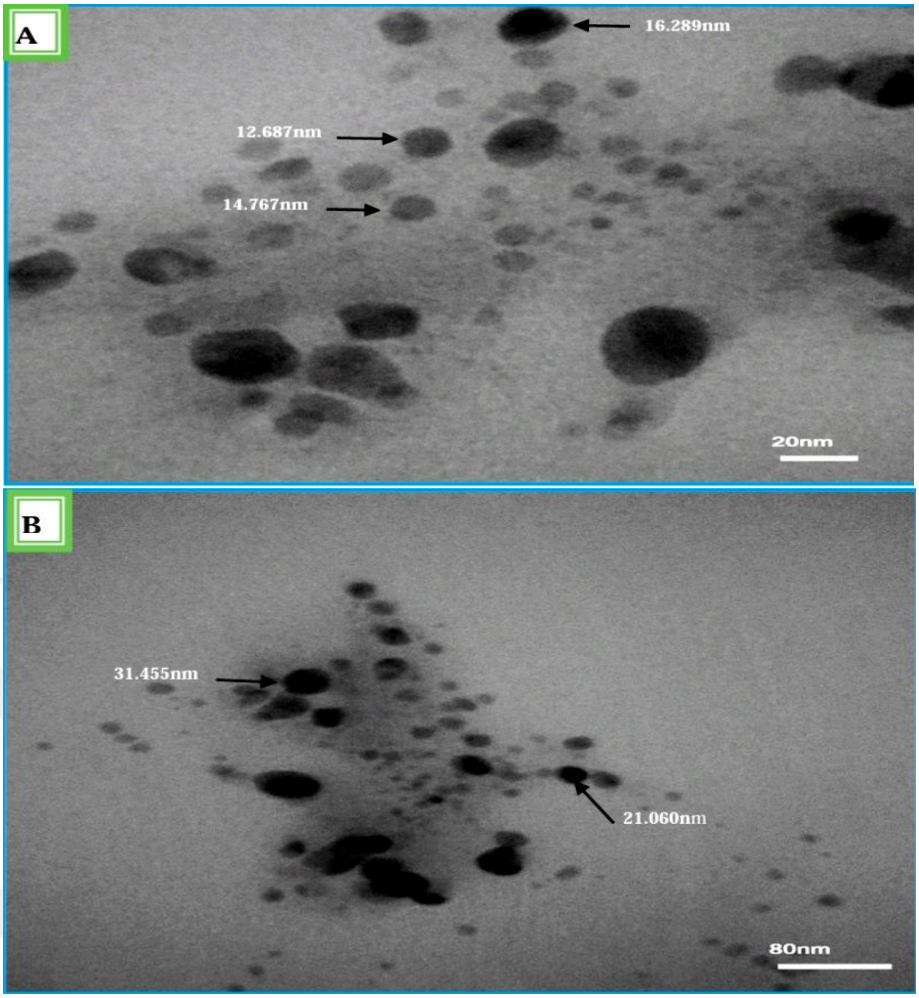

Figure 4.5 A, B, TEM analysis of biosynthesis nanoparticles

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was carried out to identify the biomolecules responsible for reducing silver ions and protein interactions synthesized from microorganisms.

The FTIR spectrum analysis showed that the supernatant of the three selected strains contained biomolecules responsible for converting silver ions into AgNPs.

The FTIR spectral analysis revealed the presence of six rings stretching vibration of Ag-BNPs at 3428.27, 2924.10, 2364.89, 1652.41, 1519.65, 1055.50 in the region of 400 – 4400 cm^{-1} (Jain *et al.* 2011) as shown inTable 4.2Figure4.6.

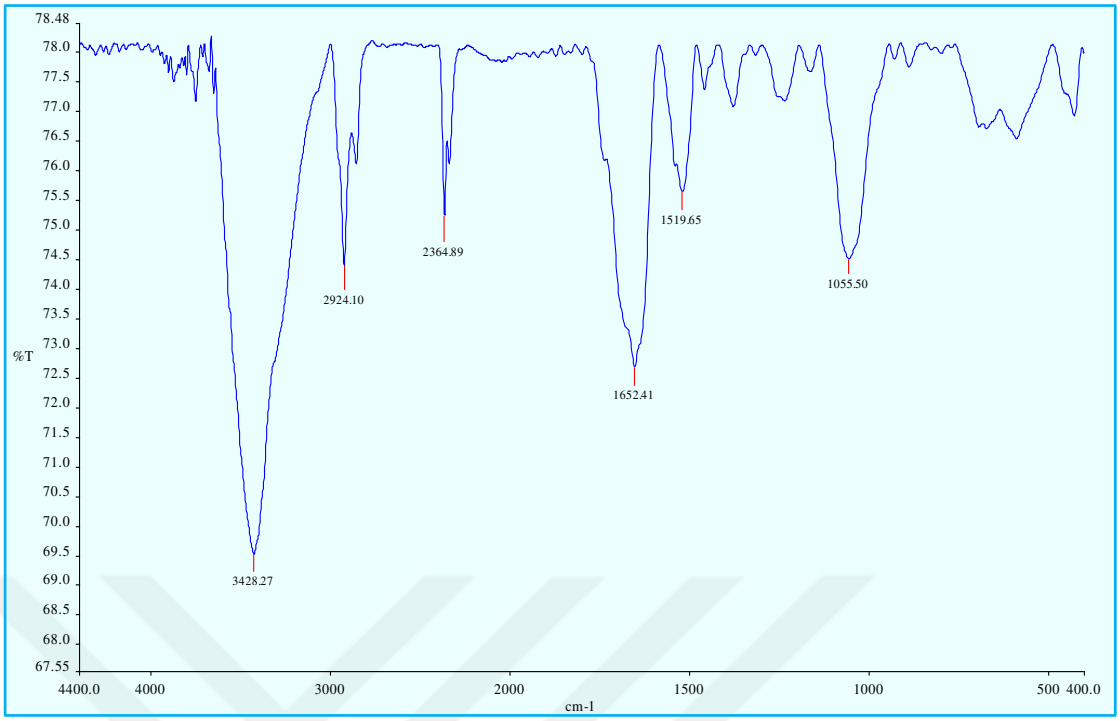

Figure 4.6 FTIR spectrum of AgNPs synthesized by isolated bacteria with distinct peaks

Table 4.2 Infection groups resulting from the FTIR analysis

Functionality groups	**Observed peak (cm^{-1})**
O-H of alcohol	3428.27
CH and CH_2 Stretching aliphatic group	2924.10
C=C Stretching of alkanes	2364.89
C=C Stretching of alkanes	1652.41
C-O	1519.65
C-O	1055.50

SEM analysis

SEM analysis indicated a substantial density of AgNPs (Figure 4.7). Significantly spherical and homogeneous AgNPs with sizes of 71.58 nm were found. The SEM image of silver nanoparticles was created by hydrogen bonds and electrostatic interactions between the bioorganic capping molecules attached to the AgNPs. The nanoparticles were not in direct contact inside the aggregates, suggesting that a capping agent

stabilized them. According to the SEM results, the larger silver particles might be the consequence of the agglomeration of smaller ones.

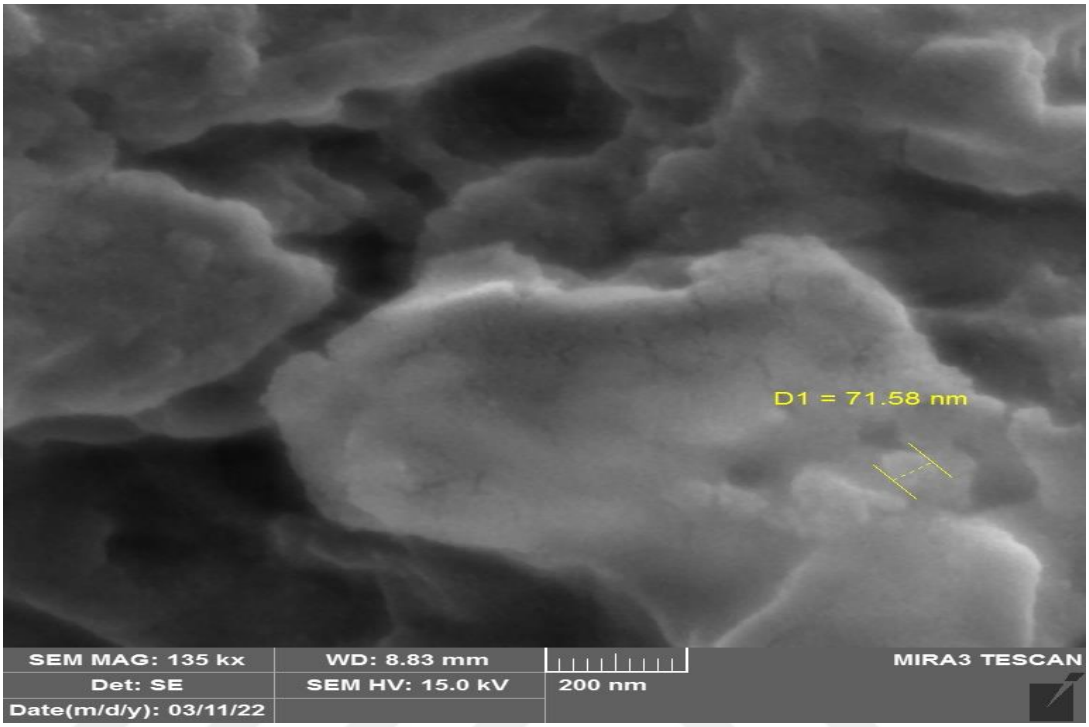

Figure 4.7 SEM analysis of biosynthesis nanoparticles

4.1.5 Molecular Study of *Leishmania*Parasite

Detection of *Leishmania*by polymerase chain reaction (PCR)

PCR was used to determine the type of parasite used in this study. TheDNA of the *Leishmania* parasite was extracted from all samples and migrated electrically to ensure its quality by the naked eye. Then it was kept at ≤-20°C in the refrigerator until it would be used.The reactions were carried out using PCR to copy a specificpart of the DNA and amplify this part numerically.

The primer for the LITSR gene and the primer for the mkDNA gene were used, and obtained a bundle of length for *Leishmania* is the ITSR gene 450bp and also obtained a bundle 120bp for *Leishmania* is the mkDNA.In this study, the PCR reaction was used

on the DNA isolated from the samples, and the products of the PCR reaction were migrated on 2% agarose gel. Hence, the studied strain showed 450bp and 120bp long bands belonging to the type *L.tropica w*here markers(Intron) were used in sizes 1000bp and 100bp (Figure 4.8).

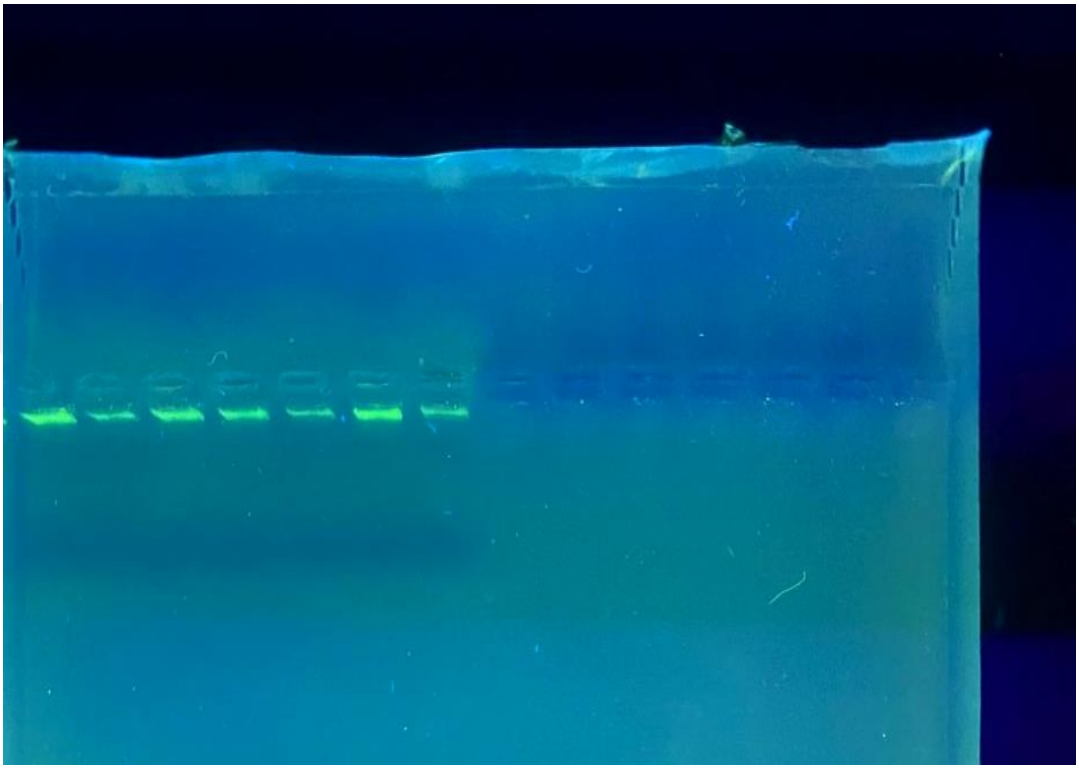

Figure 4.8 Gel electrophoresis of genomic DNA extraction from Leishmania samples, 1% agarose gel at 5 v /cm for 30 min (shows the presence of DNA)

The first primer set used in this PCR approach was from the NCBI primer design and was specific for the LITSR/L5.8S gene, with a product length of 450bp, and the PCR products were gel electrophoresis, as shown in Figure 4.9.

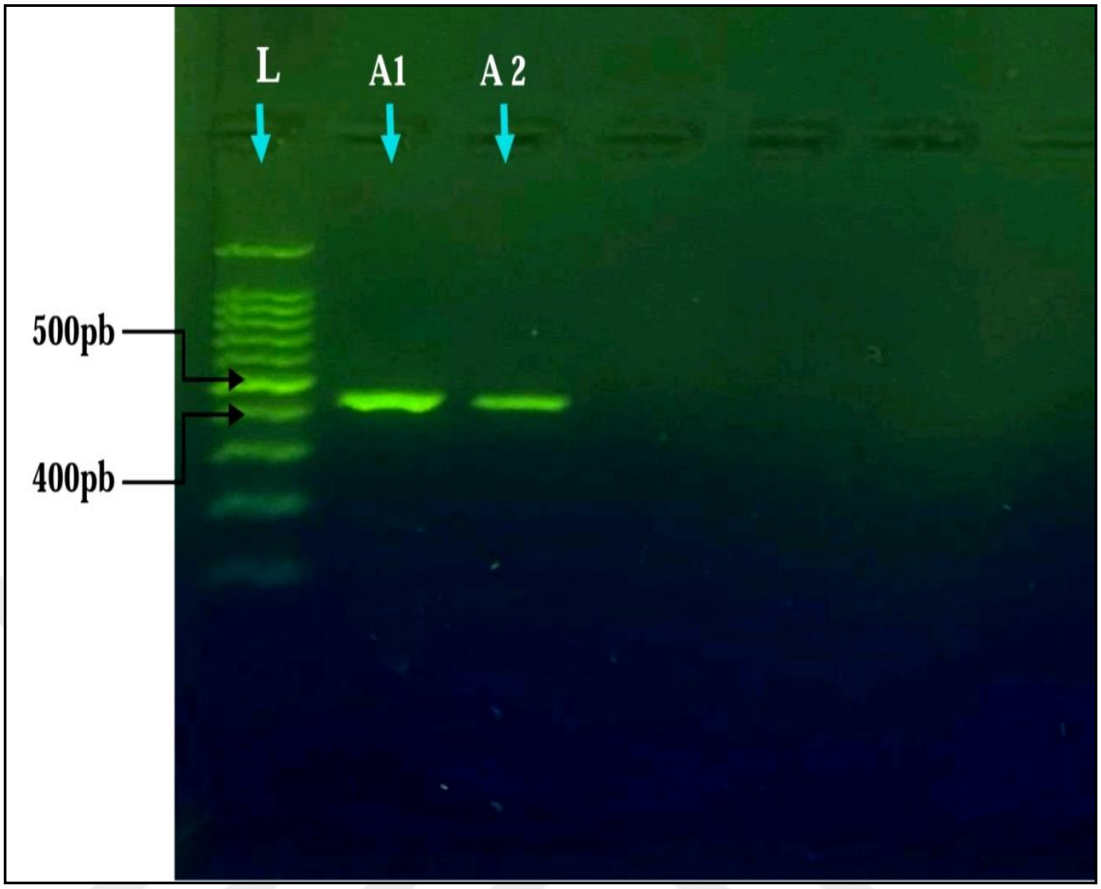

Figure 4.9 The product was electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1 hour. Lane (L) represents the molecular ladder (100)bp, and Lane (A1, A2) represents the positive PCR product size (450bp) of *Leishmania tropica*. Primer of gene LITSR/L58S

Sequencing of *18SRNA* gene

Automated DNA sequencing results in a computer output were represented by a chromatogram which is the visual representation of a DNA sequence containing nucleotide sequences and peaks. The sequencing results aligned to the sequence from the NCBI database and showed 100% complementary to the*L.tropica* under the ID:MT071547.1. The alignment summary is represented in Table 4.3.

Table 4.3*L. tropica* strains recorded in GenBank

No	Accession number	Closed species	Sequence identity with reference strain (%)
1	MT071547.1	*Leishmaniatropica*	100%

The alignment of *L. tropica* to the NCBI sequence showed 100% complementary to the region of about 206bp with a score of 150 bits to the plus strand. Figure4.10 shows the alignment results and the obtained sequence of this study was submitted to the NCBI database, and accession numbers were received; ON241029. *Leishmaniatropica* strain AA1 internal transcribed spacer 1, partial sequence, 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

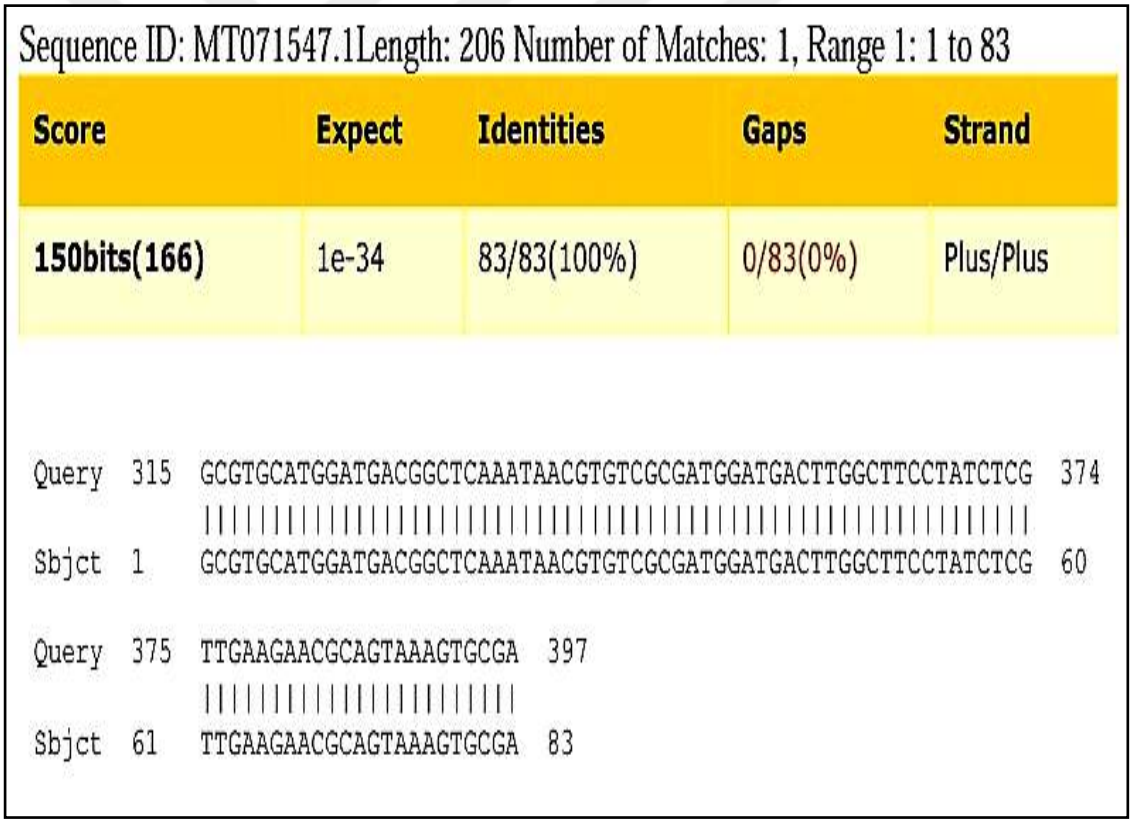

Figure 4.10 Sequence alignment showing 100% complementary to *L. tropica*

The product length of the second primer set employed in the mkDNA gene-specific PCR technique was 120bp, and the PCR products were gel electrophoresis, as shown in Figure 4.11. Due to the 120 base pair short, no sequences were transmitted.

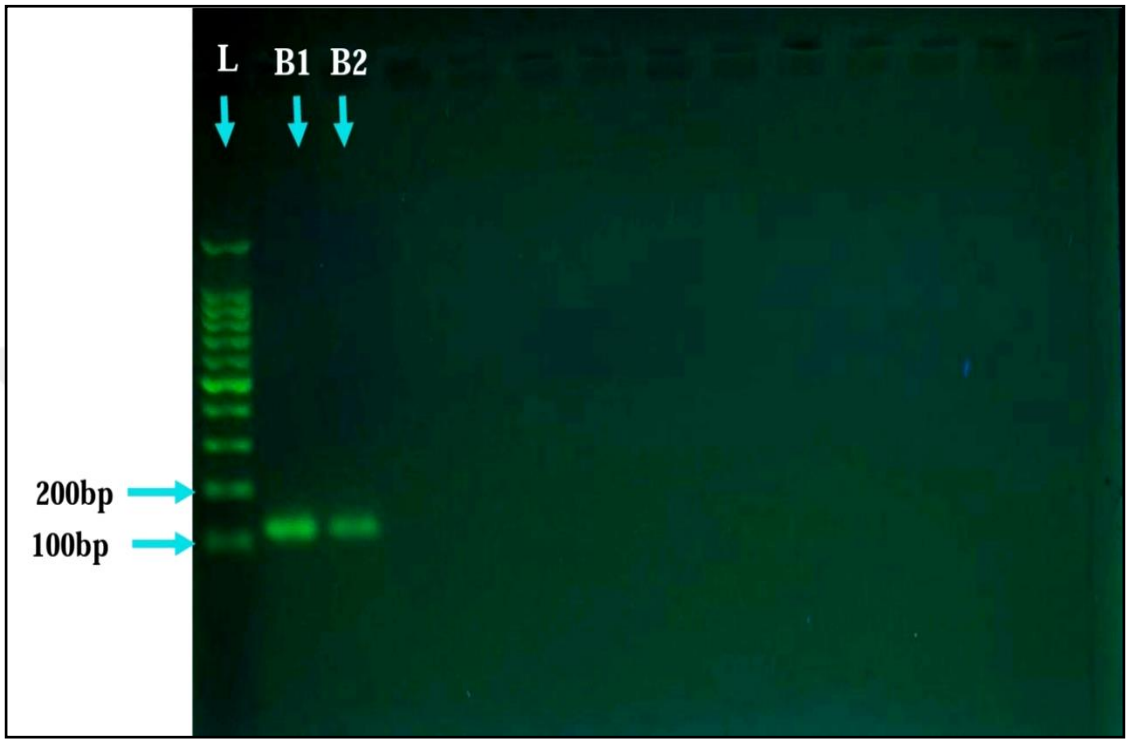

Figure 4.11 The product was electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1 hour. Lane (L) represents the molecular ladder (100)bp, and Lane (B1.B2) represents the positive PCR product size (120bp) of *Leishmania tropica*. Primer of gene mkDNA

Ant parasiticactivity of AgNPson*L.tropica*

The strains selected to carry out the biosynthesis of silver nanoparticles were *Pseudomonas aeruginosa* strain ALK320, *Bacillus cereus* strain HNB5, and*Corynebacteriumstriatum* strain 1910ICU141.

Multiple concentrations of chosen silver nanoparticles were added in this stage (400, 200, 100, 50, 25, and 12.5 µg/mL) for each sample. Following the incubation period,the optical densities of the parasites with the indicated concentrations were measured in triplicates at 620 nm at 27°C for 24 hours.

After 24 hours of incubation, the antiphrastic activities of extracellular AgNPs on *L.tropica* showed the highest inhibition (64.72 %) with 400 µg/mL. This implying that only 35.28 % were able to form antiparasitic activity in soluble formazan products. They remained as alive parasites (viability rate decreased, which means that the rate of grown parasites will be inhibited, so they could not convert MTT compound and form insoluble formazanproducts). On the other hand, the lowest inhibiting was recorded 1.93 %, zero, and 1.06% of parasites, respectively, as shown in Figure 4.13. This indicated that as the concentration of AgNPs rose, so did its toxicity. The results showed that AgNPs strongly suppressed parasite proliferation (64.72%).

The Cytotoxicity of Ag NPs on the *Leishmania* parasite by MTT

The cytotoxicity effect of the Ag NPs against*L.tropica* was investigated by MTT assay. Parasite viability was based on a dose-dependent response and decreased by increasing the Ag NPs concentration. In other words, the maximum toxicity was related to a concentration of 400 µg/ml after 24 h.

The effect of AgNPs on the growth of*L.tropica*

The effects of different concentrations of AgNPs on the number of *Leishmania* parasites were investigated after incubation for 24 h.In this test significant differences were observed between all concentrations of AgNPs and control groups ($P < 0.05$). The results showed that by increasing the concentration of Ag NPs, the proliferation of*L.tropica* decreased significantly. Concentrations of 400, 200, and 100 µg/mL Ag NPs with 24 h incubation times showed the most effectiveness, while 50, 25, and 12.5 µg/mL concentrations after 24 incubation presented the least effective in inhibiting the reproduction and movement of *L. tropica*. Moreover, it was found that a high concentration (400 µg/mL) of Ag NPs inhibited the proliferation of *L.tropica* by 64.72 % after 24 hours.

Metabolic activity (viability)

The metabolic activity of *L. tropica*parasites reduced as AgNP concentrations increased. It was clear that each concentration of Ag-NPs had a detrimental effect on the metabolic activity of *L. tropica* compared to the control group. Consequently, at a concentration of 400µ g/mL, AgNPs had the greatest suppressive impact on parasites. The study's findings on the impact of AgNPs on the metabolic activity of *L. tropica*revealed that as AgNP concentrations increased, the cell viability values declined progressively Table 4.4 and Figure 4.12.

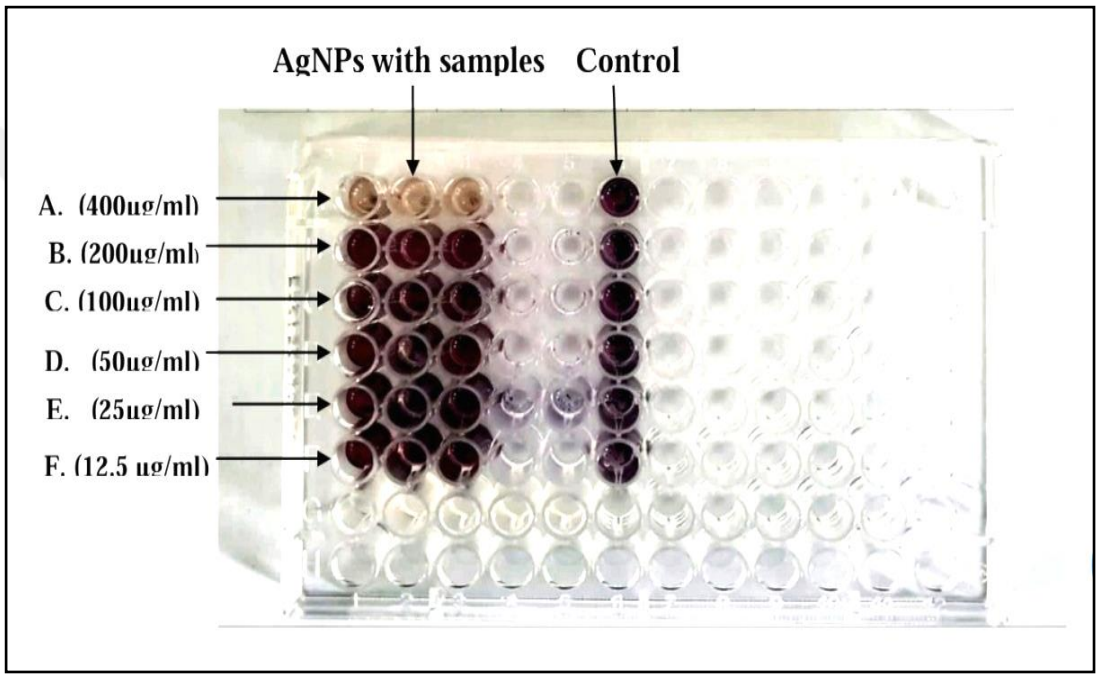

Figure 4.12 96 - Well microliter plate

Table 4.4 Results of the effect of Ag-NPs on the growth of *Leishmania parasites* after

No	Concentration Ag-NPs	Percentage of live cells	Percentage of dead cells
A	400 µg/mL	35.28 %	64.72 %
B	200 µg/mL	70.21 %	29.79 %
C	100 µg/mL	74.53 %	25.47 %
D	50 µg/mL	98.94 %	1.06 %
E	25 µg/mL	100 %	Zero
F	12.5 µg/mL	98.07 %	1.93 %

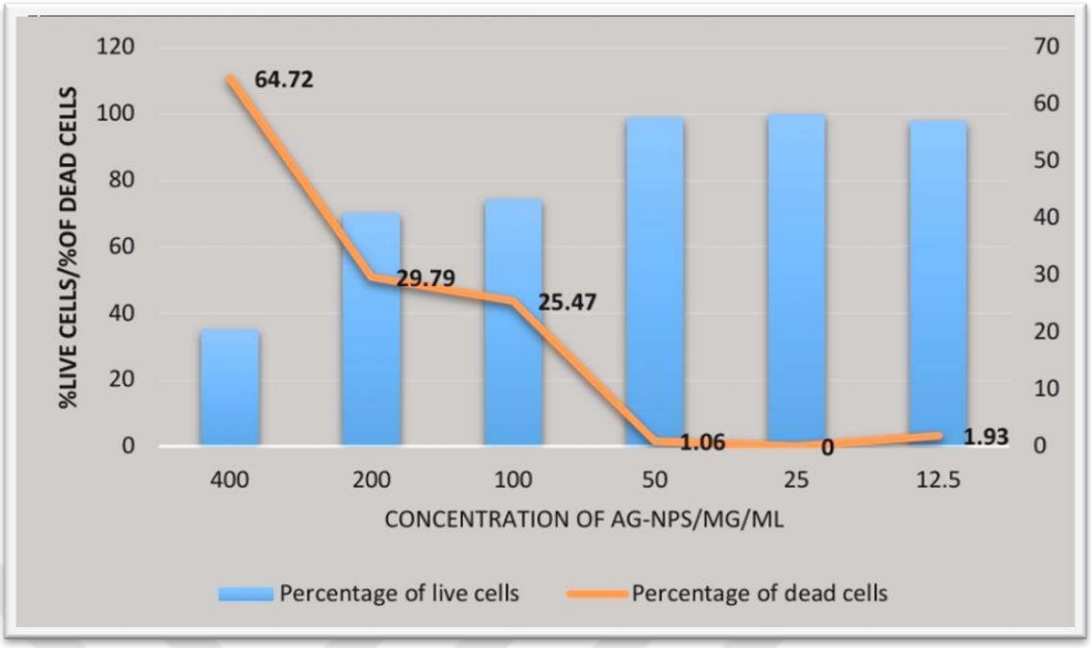

Figure 4.13 Mean and standard deviation of *L. tropica* (1×10^4) Cultured with different concentrations of AgNPs in comparison with control groups for 24h

4.2 Discussion

Silver nanoparticles were synthesized using cell-free supernatants of*P.aeruginosa*strain ALK320, *B.cereus* strain HNB5 and *C. striatum* strain 1910ICU141. This is consistent with a study on production of silver nanoparticles by *P.stutzeri* AG259 from a silver mine (Punjabi *et al*. 2017). Numerous studies have indicated that silver nanoparticles were synthesized by bioreduction silver nitrate with a culture supernatant of *B.licheniformis*(Sarangadharan and Nallusamy 2015). Furthermore, the extracellular silver nanoparticles synthesized culture supernatant of *P.aeruginosa*(Paul and Sinha 2014). In the present study, bacteria were screened to determine the production of nitrate reductase (Figure 3.2). Generally, one of the most widely accepted mechanisms for silver biosynthesis is the presence of the nitrate reductase enzyme, which converts nitrate into nitrite(Bhusal and Muriana 2021).

AgNPs were effective on *L. tropica* and had a reverse correlation with its concentration. According to the anti-Leishmania assay results, this nanoparticle's concentrations were

400, 200, and 100 μg/mL after 24 h. AgNPs caused programmed cell death (PCD) in *L. tropica* and showed 64.72%, 29.72%, and 25.47% of apoptosis, respectively.

In another study, the effect of silver (Ag) NPs at the presence of ultraviolet (UV) light led to a reduction in the metabolic activity and proliferation rates of *Leishmania tropica* (Allahverdiyev *et al*. 2011).

It is worth noting that this study is compatible with a study presented by (Mohammadi *et al*. 2021) who study the effect of AgNPs on *L. major* and found the maximum inhibition was 60.18% at a concentration of 400 μg/mL .

The researchers state that nanoparticles can enter the cell directly through the pores in the cell membrane or through ion channels and transporter proteins on the plasma membrane. Endocytosis is a process by which nanoparticles enter cells. They discovered that nanoparticles entered into the cell could interact directly with oxidative organelles like mitochondria. Furthermore, Ag ions interacted with the disulfide or sulfhydryl groups of enzymes, disrupting metabolic pathways and ultimately leading to cell death (Adibkia *et al.* 2010).

Silver nanoparticles can produce reactive oxygen species (ROS), which Leishmania parasites are susceptible to it. Nanoparticles can act on these soft bases and destroy DNA, leading to cell death. AgNPs decreased parasites' metabolic activity and proliferation values compared to the control groups(Ahmad *et al*. 2020).

In the present study, the polymerase chain reaction (PCR) technology was mainly relied on in the molecular diagnosis of bacteria and parasites used for the study. The reason for this is that the polymerase chain reaction (PCR) technology is a laboratory technology that is currently a mainstay of molecular biology,and the elegant simplicity of interaction and the relative ease of practical processing steps. Indeed, together with relevant bioinformatics sources for designing oligonucleotide primers and determining desired experimental conditions, they provide a rapid means of DNA identification and

analysis (Fida *et al*. 2021).PCR technique is characterized by its high accuracy due to the quality of the bases used in the reaction, which binds to a specific place of the studied DNA, with very little extracted DNA (up to 50 Nano grams). Some studies have relied on this technique to detect visceral or cutaneous leishmaniasis by analyzing peripheral blood samples, although they contain a small number of parasites (Tawfeeq and Ali 2022).

The ITS1-PCR assay identified the studied *Leishmania*species. However, the present study is consistent withMouttaki *et al*. (2014)'s study which indicated the molecular diagnosis of pathogenic *Leishmania* species in Morocco using three PCR-based assays.

Jariyapan*et al.* (2021) noted the determination of the sequence of nitrogenous bases in DNA andlocation of the gene as a diagnostic characteristic of the *Leishmania* parasite(Jariyapan *et al*. 2021).Many researchers have performed molecular diagnostics through this technique, confirming the high sensitivity of the diagnosis (Sundar and Singh 2018).

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

1- The PCR-based assays on the samples increased the speed and sensitivity of diagnosing bacteria and parasites compared to conventional techniques.

2- Soil is a rich source of bacteria to produce silver nanoparticles.

3- The current study provides an environmentally friendly and cost-effective approach for synthesizing powerful silver nanoparticles (biologically) against pathogenic parasites. This may prove to be a viable alternative to traditional antibiotics.

4- The antiparasitic effects of extracellular AgNPs on *L.tropica* yielded the best results, with AgNPs significantly suppressing parasite multiplication.

5.2 Recommendations

- Advanced molecular techniques such as real-time PCR should be used to examine DNA sequences in future studies. Real-time PCR can also be used to examine the molecular characterization of different *Leishmania* species.

- It is necessary to conduct a comprehensive study to find out the mechanism of the action of silver nanoparticles on the DNA of pathogenic parasites.

- Further studies are recommended to be conducted on the use of AgNPs to eradicate other organisms such as bacteria, parasites, fungi, and algae.

- Other studies should be conducted by biosynthesis producing new metal nanoparticles such as Gold, Zinc, and Copper.

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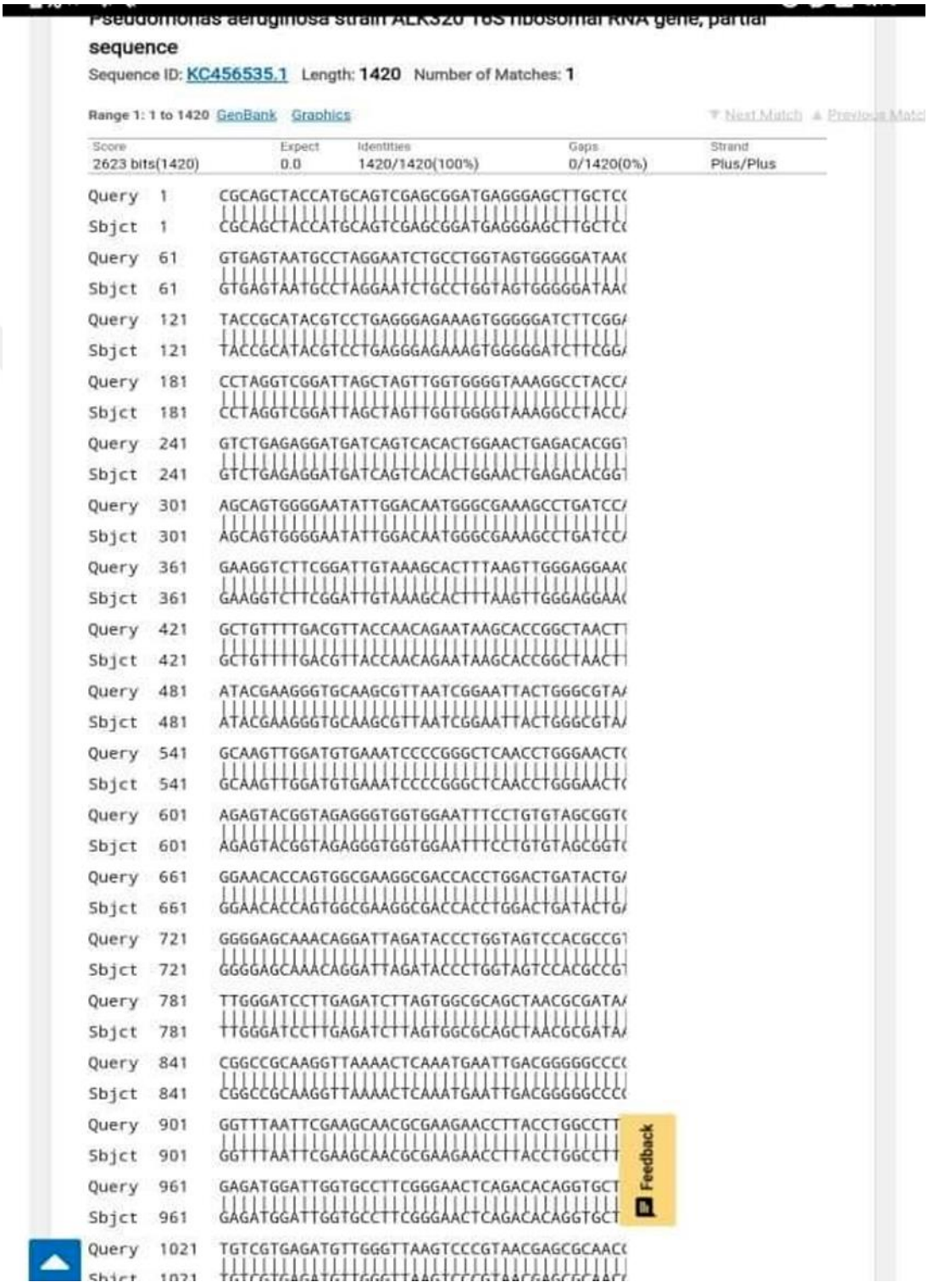
APPENDICES

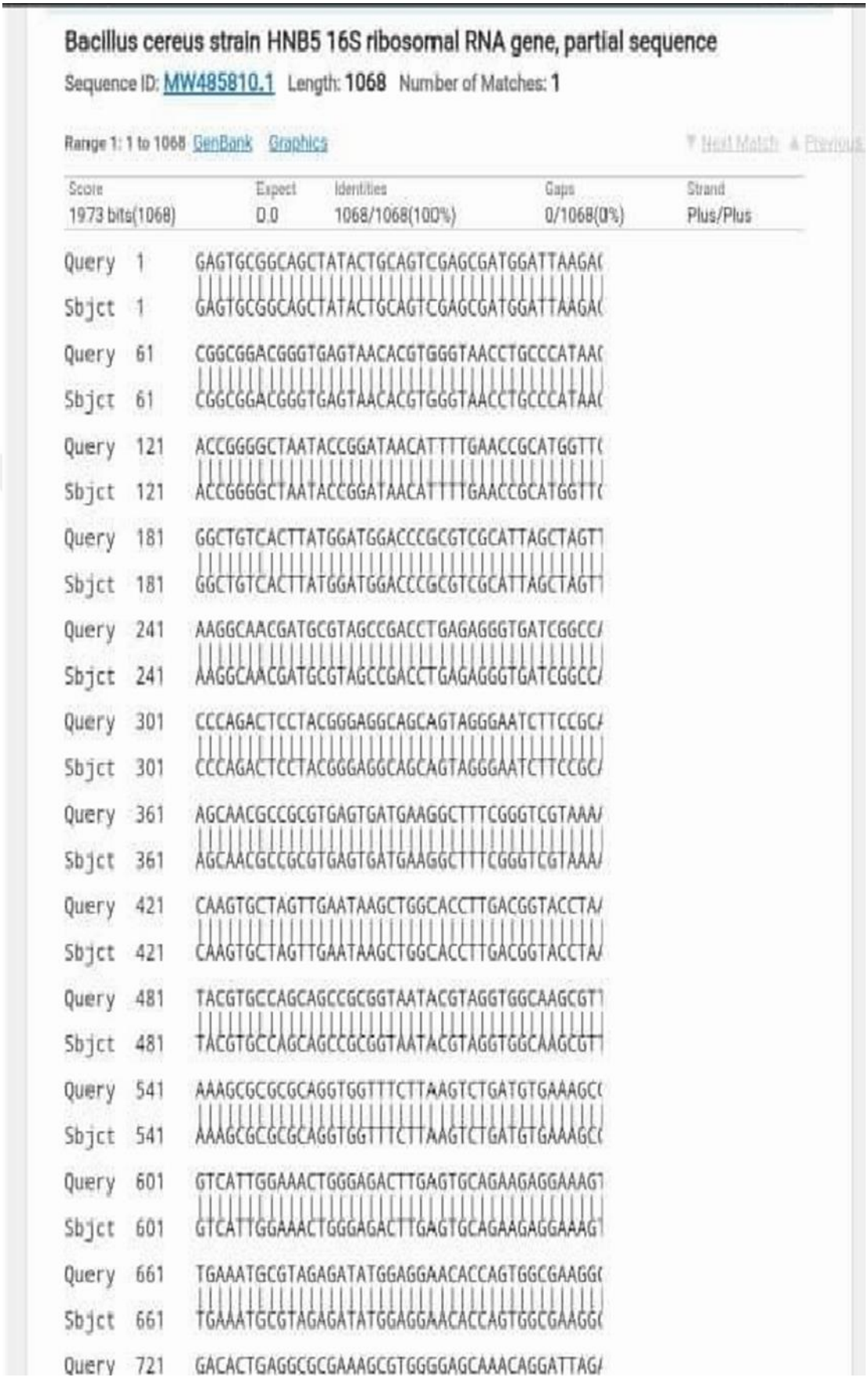
APPENDIX1.Facilitating the task

APPENDIX 2. A pairwise alignment of the strain's *16s rRNA* gene sequence, *Pseudomonas aeruginosa* strain ALK320, *Bacillus cereus* strain HNB5 and *Corynebacterium striatum* strain 1910ICU14, was performed using NCBI online blast

APPENDIX 3. The obtained sequences were submitted to the NCBI database, and accession numbers were received; ON340727, ON340740, ON340743, and ON241029

APPENDIX2.A pairwise alignment of the strain's *16s rRNA* gene sequence, *Pseudomonas aeruginosa* strain ALK320, *Bacillus cereus* strain HNB5 and *Corynebacterium striatum* strain 1910ICU14, was performed using NCBI online blast





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Corynebacterium striatum strain 1910ICU141 16S ribosomal RNA gene, partial sequence

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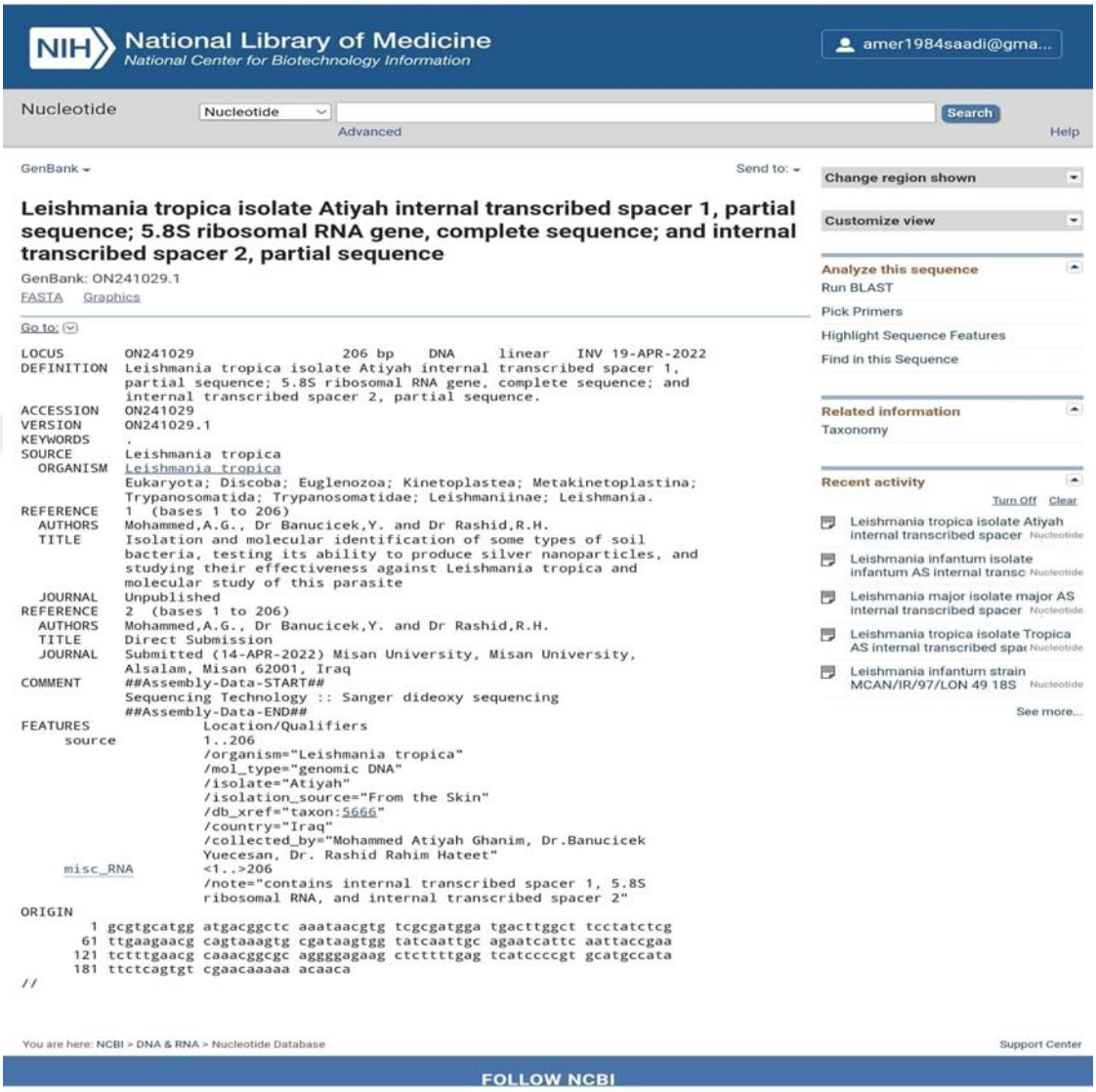
APPENDIX 3. The obtained sequences were submitted to the NCBI database, and accession numbers were received; ON340727, ON340740, ON340743, and ON241029



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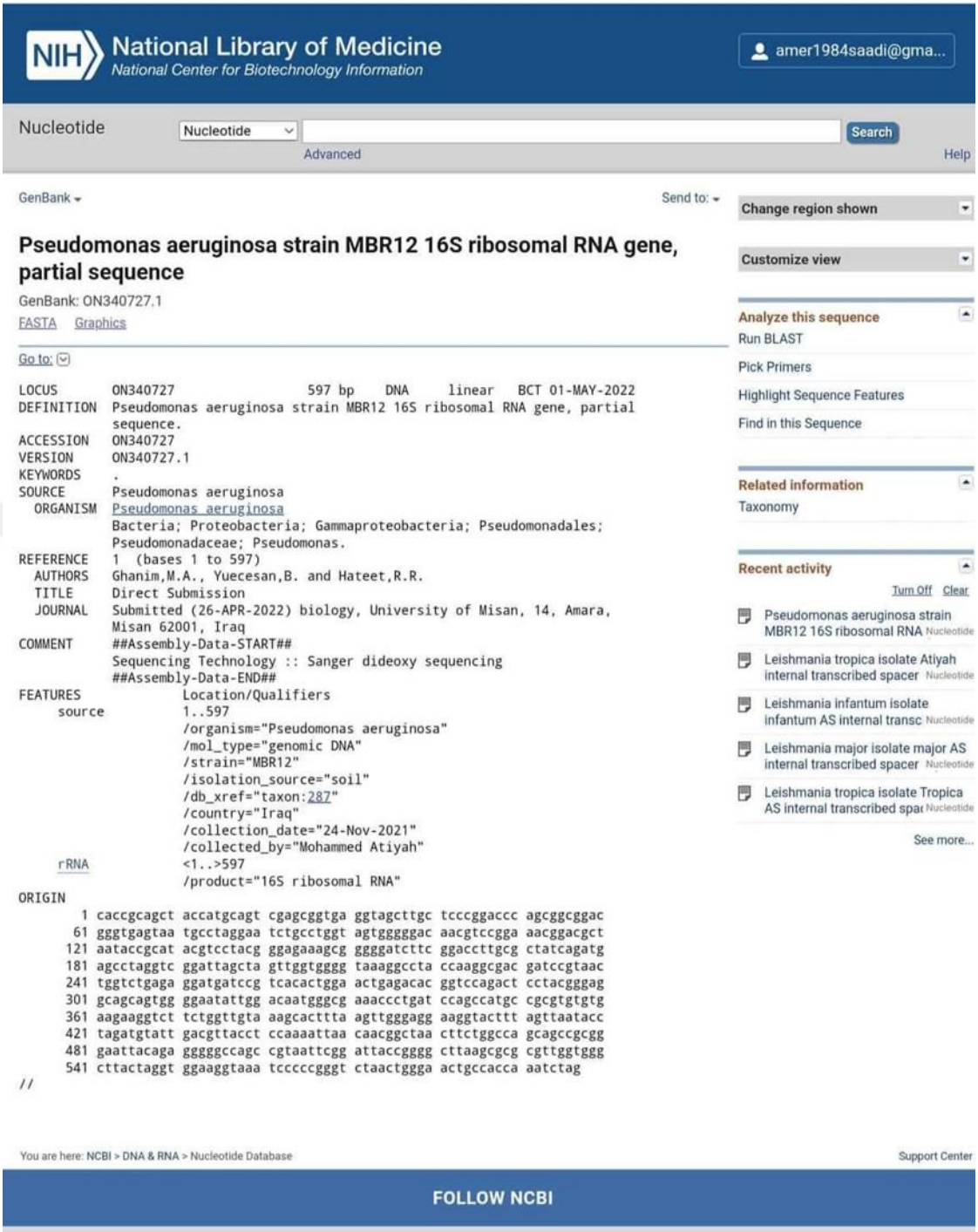
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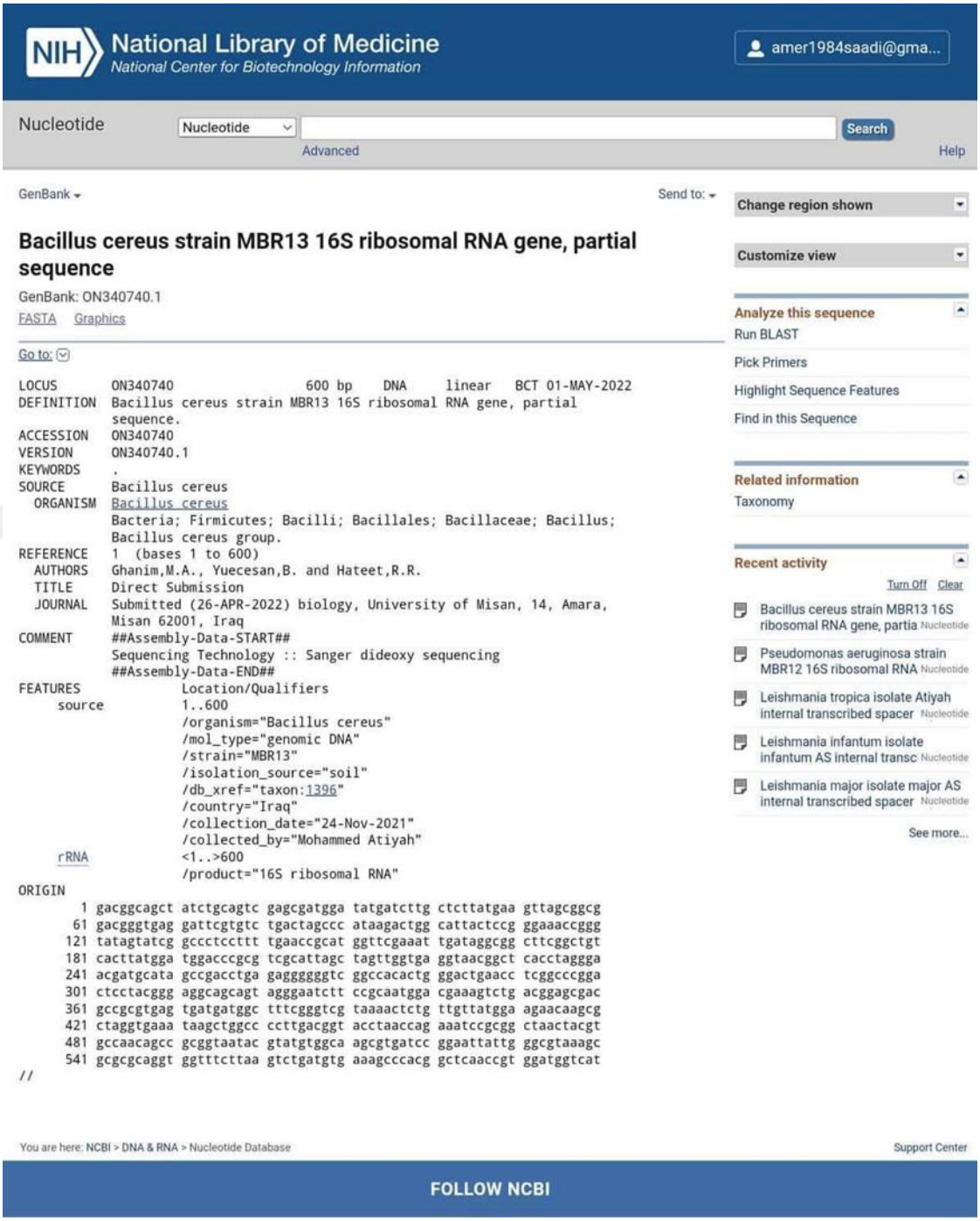
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AUTHORS Ghanim,M.A., Yuecesan,B. and Hateet,R.R.
TITLE Direct Submission
JOURNAL Submitted (26-APR-2022) biology, University of Misan, 14, Amara, Misan 62001, Iraq
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Pseudomonas aeruginosa strain MBR12 16S ribosomal RNA Nucleotide
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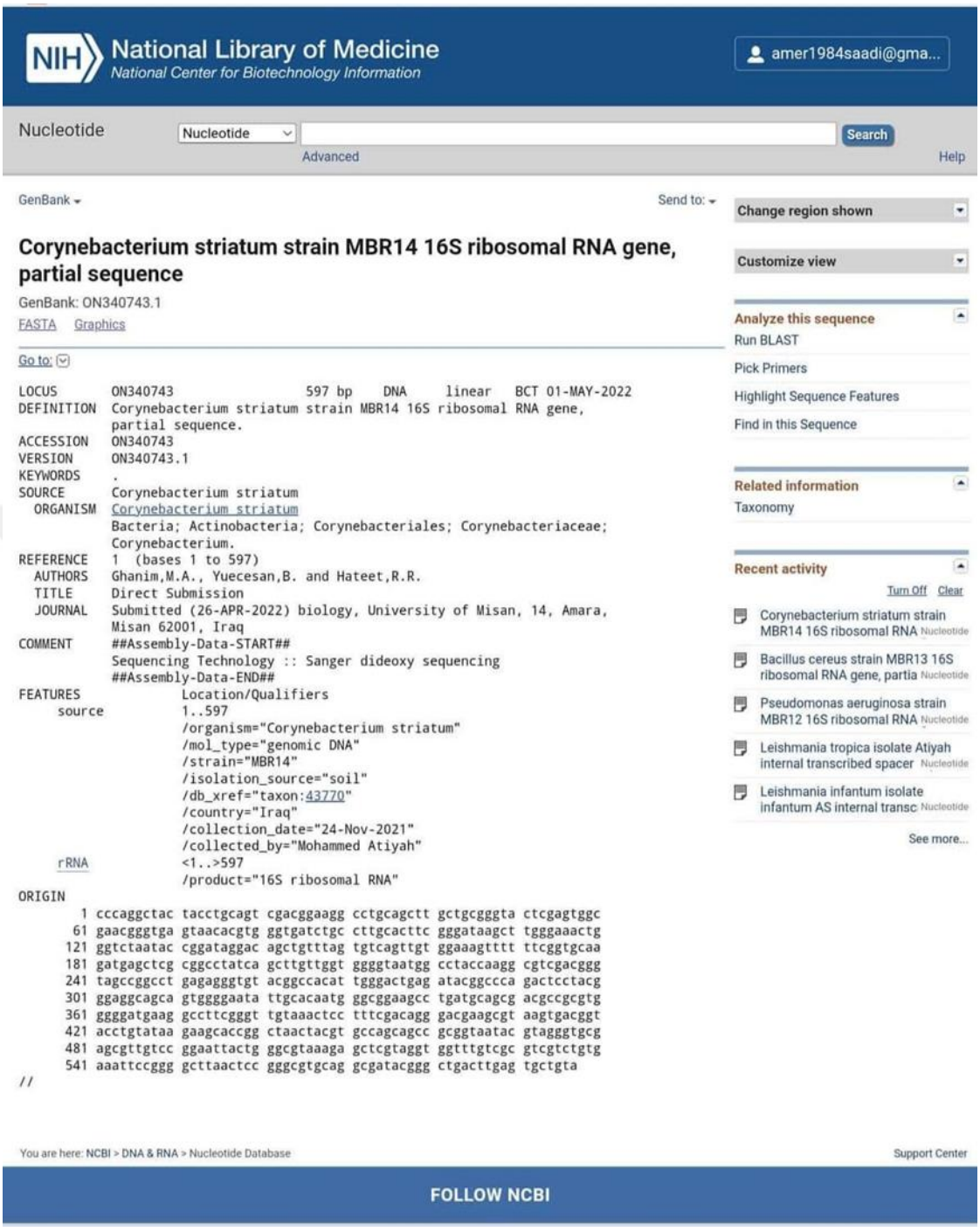
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Bacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium.
REFERENCE 1 (bases 1 to 597)
AUTHORS Ghanim,M.A., Yuecesan,B. and Hateet,R.R.
TITLE Direct Submission
JOURNAL Submitted (26-APR-2022) biology, University of Misan, 14, Amara, Misan 62001, Iraq
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Bacillus cereus strain MBR13 16S ribosomal RNA gene, partia Nucleotide
Pseudomonas aeruginosa strain MBR12 16S ribosomal RNA Nucleotide
Leishmania tropica isolate Atiyah internal transcribed spacer Nucleotide
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Education

MSc	Çankırı Karatekin University Graduate School of Natural and Applied Sciences Department of Biology	2020-Present
Undergraduate	Al Basra University Facultyof Sciences Department of Biology	2003-2007