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Antibacterial and Anticancer Activities of *Hirudo medicinalis* Salivary- Mediated Silver Nanoparticles

A dissertation

Submitted the Council of the College of Science at University Babylon in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy In Science / Biology

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

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إِنَّمَا يَخْشَى اللَّهَ مِنْ عِبَادِهِ الْعُلَمَاءُ إِنَّ اللَّهَ عَزِيزٌ غَفُورٌ﴾

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Dedication

Praise be toAllah, it is enough, and prayers be upon the beloved Chosen One and whoever fulfilled it…

As for after, I dedicate this humble effort to ***Imam Al-Hijjah***, may Allah hasten his honorable reappearance…

To the one who honored me with bearing his name, ***my father***, may Allah have mercy on him…

To the one who misses the warmth of her applause at this moment, and I do not miss her prayers whose fruits I reap every moment, ***my mother***, may Allah have mercy on her…

To the support, humerus and forearm, ***my husband, my daughters, my brothers, my sisters, my colleagues***.

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Summary:

Leech ability to displace antibacterial activity had attracted the attention of traditional therapists, physicians and researchers. Leech saliva and fatty acids are important constituents and commonly possess antimicrobial activities.

The biological method for the synthesis of nanoparticles occupies an important area due to economic and ecofriendly benefits when compared with physical and chemical synthesis methods. Hence ,this study aims to evaluate the antibacterial activity of crude leech salivary extract, leech oil extract and leech salivary extract-mediated silver nanoparticles against some pathogenic bacteria and determination the cytotoxicity of LSE-AgNPs on Hepatocellular carcinoma human cell line.

Leech saliva collected after twelve weeks starvation and sucking the phagostimulatory solution (saline 0.15 M + Arginine 0.001 M) from (80) leeches. Crude oil extract was collected from (20) leeches using Clevenger machine based on the distillation system. The LSE-AgNPs is biologically synthesized, Characterized by Dynamic Light Scattering and Field Emission Scanning Electron Microscope. CLSE, LSE-AgNPs and crude leech oil extract were screened for its antibacterial activities against American Type Culture Collection bacteria *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Streptococcus pyogenes* species using agar well diffusion method, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration test. The in-*vitro* cytotoxic effects of LSE-AgNPs are screened against HepG2 cell line and the percentage of cell inhibition was confirmed by Modular Test Tool Kit (MTT) assay.

CLSE and crude leech oil extract were inactive on all the test isolates at ($P \geq 0.05$). Standard antibiotics (Amikacin) used as the positive control inhibited the growth of the test isolates with inhibition zone size (25.00±4.100mm for *S.aureus,* 23.00±4.400mm for *E.coli,* 22.00±4.700mm for *P. aeruginosa*) and 25±5.800mm for *S.pyogenes* by (Ampicillin) respectively.

Biosynthesized, LSE-AgNPs are linked to a shift in colorless solution to brown that lasts for 48 hours via *Hirudo medicinalis.* Saliva act as reducing and steadying agents. DLS results of LSE-AgNPs size is 649.1 nm and their zeta latent is -0.060. FE-SEM was used to take image from the surface of the samples. Images were taken at a magnification of 10 KX (10.000 x and 35 KX). The samples are containing the particles well dispersed and almost have square shapes. The sizes of the nanoparticles were in the range of about 20 to 720 nm with an average value of 600 nm. The test was performed with a confidence level of 95%.

The LSE-AgNPs (15µl) in current study inhibited the growth of ATCC bacteria *S.aureus, S.pyogenes, E. coli* and *P. aeruginosa* in different concentrations 100,200,300 and 400µg/ml compared with Ampicillin and Ceftriaxone as positive control. According to the antibiogram results found that the suitable concentration of LSE-AgNPs is 100µg/ml, from this concentration the dilution for MIC test were prepared.

MIC test results found (10µl) of LSE-AgNPs from high concentration(100µg/ml) to low concentration(3.13µg/ml) respectively showed in comparison with antibiotics and negative control. The results reveal significant differences with different concentrations of Ag NPs for each isolates of this study, but there were no significant differences between the 100µg/ml, 50µg/ml

concentrations of LSE-AgNPs compared with an antibiotic and negative control at $p \leq 0.05$ for all bacterial isolates.

The MIC and MBC of LSE-AgNPs are assayed using micro broth dilution method. The MIC for *P. aeruginosa*, *E. coli,* and *S. aureus* respectively was (50µg/ml) whereas MIC for *S. pyogenes* was (25µg/ml). MBC for *P.aeruginosa, E. coli*, and *S. aureus* respectively was (100µg/ml) respectively whereas MBC for *S. pyogenes* was (50µg/ml).

The viability of $HepG_2$ reduce after interaction with various concentrations of LSE-AgNPs. This reduction increase gradually between (24 and 48 hrs.) for the same concentration exposure to LSE-AgNPs.

The liver ($HepG_2$) cancer cells is cultured and its morphology detail was observed via light microscopy after incubation for 24, 48 and 72hrs in the presence of varying concentrations (25, 50, and 100µg/ml) of LSE-AgNPs. Representative images of cell lines show remarkable changes in the morphology at LSE-AgNP concentrations of 25 and 50µg /ml. At 48 hours, this nanoparticle's IC_{50}(0.0124) value was 50µg /ml. The morphology of cells at these concentrations was greatly affected.

The results obtained suggest that LSE-AgNPs could be used in treating infectious diseases caused by *P. aeruginosa and S. pyogenes*, *E. coli*, and *S. aureus*.

According to the MTT and MBC assays, it is revealed that the inhibitory concentrations of LSE-AgNPs for selected bacteria strains are toxic for HepG2 cell lines.

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List of Abbreviations

Abbreviations	Meaning
1D	One Dimensional Gel Electrophoresis
$AgNO_3$	Silver Nitrate
Ag-NP	Silver Nitrate Mediated Nanoparticles
AM	Ampicillin
AMPs	Antimicrobial Peptides
AN	Amikacin
ASM	American society for Microbiology
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLS	Crude Leech Saliva
CLSI	Clinical and Laboratory Standard Institute
CRO	Ceftriaxone
D.W	Distilled Water
DIC	Diffuse Intravascular Coagulation
DLS	Dynamic Light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSR1	Desk Sputter Coater
ELISA	Enzyme Linked Immunosorbent assay
EW-AgNP	Earth Worm-AgNPs
FBS	Fetal Buffer Solution
FDA's	United States Food and Drug Administration
FE-SEM	Field emission scanning electron microscopy
H. m.	*Hirudo medicinalis*
$HEPG_2$(HCC)	Hepatocellular carcinoma
HPLC	High Performance Liquid Chromatography
hrs.	Hours

IC_{50}	50% Inhibition Concentration
LSE	Leech Saliva Extraction
LSE-Ag	Leech Saliva Extraction Mediated Silver Nanoparticles
M.H.A	Mueller Hinton Agar
M.H.B	Mueller Hinton Broth
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MTT	3-2-5-diphenyltetrazolium-bromid, Modular Test Toolkit
NCBI	National Center for Biotechnology Information
NPs	Nanoparticles
NS	Normal Saline
OD	optical density
PBS	Phosphate- buffered saline solution
R	Resistance
ROS	Reactive Oxygen Species
S	Sensitive
VEGF	Vascular Endothelial Growth Factor

List of Units

Abbreviations	Meaning
µg	Microgram
µg/ml	microgram for milliliter
µm	Micrometer
Kx	Kilo(x), magnification bower
mM	Milimolary
Mm	Millimeter
Nm	Nanometer

Chapter One
Introduction

1. Introduction

People have employed a range of plants and animals to both prevent and treat illnesses since the dawn of time. A conventional form of treatment called hirodotherapy uses medicinal leeches (Becanim *et al*., 2022). There are more than 600 different species of leeches known to exist. Only a small number, including *Hirudo medicinalis*, are utilized in medicine. Medical leech therapy is now mostly employed in plastic and reconstructive microsurgery, with new and intriguing prospective therapeutic uses in many other disorders (Montinari and Minelli, 2022). This species thrives in temperate environments, and murky freshwater ponds with weed growth are ideal habitats for it (Singh and Shukla, 2022). They are annelid worms known as sanguivorous or bloodsuckers, and they have become increasingly popular in dentistry in addition to medicine.

Non-antibiotic treatment methods are used in conjunction with these polypeptoides-based bactericides to combat lethal bacterial strains brought on by biofilm formation and acquired antibiotic resistance. These non-antibiotic treatment methods have the ability to elude mechanisms related to acquired antibiotic resistance and to reduce the emergence of drug resistance (Zheng *et al*., 2023). Since the pathogen needs to alter the cell membrane's structure and electrophysiology to counteract the peptide, the likelihood of establishing Antimicrobial peptides resistance is minimal (Lai *et al*., 2023).

It has been used to treat a variety of infectious disorders in traditional medicine. The majority of organisms' cells are made up of lipids and fatty acids, which serve as metabolites, storage products, membrane components, and energy sources that may mediate the body's chemical defense against microbes (Dziekonska *et al*., 2009; Nelson and Graf, 2012).

The United State Food and Drug Administration's approval of the use of leeches in plastic and reconstructive surgery in 2004 and the Turkish Ministry of Health's approval of leech therapy in the treatment of some diseases with the Regulation on Traditional and Complementary Medicine Practices in 2014 both marked a real turning point in the use of leeches in modern medicine (Karatas *et al*.,2022). The site-biting, blood suction, and—most significantly—the injection of leech saliva into the site, which includes numerous bioactive chemicals, are all associated to the therapy properties of leeches (Koeppen *et al*., 2020). Leech saliva contains compounds with anti-inflammatory, anticoagulant, platelet inhibitory, thrombin regulating, analgesic, extracellular matrix degradative, and antibacterial properties (Özkaya, 2023). A total of seven species (*Hirudo medicinalis, Hirudo verbana, Hirudo orientalis, Hirudo troctina, Hirudo nipponia, Hirudo sulukii and Hirudo tianjinensis*) have been described in the genus Hirudo. Leeches in the genus Hirudo are distributed in Europe, Asia, and North Africa (Wang *et al*., 2022). In addition to this, the North American Medical Leech is also less efficient (Elliott and Kutschera, 2011).

Nanotechnology is widely used in the realm of medicine as nanomedicine. There may be uses for some nanoparticles in cutting-edge diagnostic tools, imaging and methodology, pharmaceutical items, biomedical implants, and tissue engineering. Nanotechnology now allows for the safer administration of high-toxicity medicines like chemotherapy for cancer. Additionally, wearable technology can identify critical alterations in vital signs, cancer cell states, and infections that are actually occurring in the body (Haleem *et al*., 2023).

These particles are found in the range of 1–100 nm, along with the unique biomedical properties that differentiate them from the bulk elemental form (Shah *et al*., 2015; Abdelghany *et al*., 2018). The small size of NPs gives them a larger

surface-to-volume ratio, making them more beneficial in biochemical and catalytic activity than other particles with the same composition (Jyoti *et al*.,2016; Rafique *et al*.,2017).

AgNPs are primarily employed in antibacterial and anticancer treatment, but they are also used as biosensors, vaccine adjuvants, anti-diabetic agents, and in the promotion of bone and wound healing. The primary biological effects of AgNPs are the release of silver ions (Ag+), production of reactive oxygen species (ROS), and degradation of membrane structure (Xu *et al*., 2020). Currently, nanoparticles (NPs) are created utilizing a variety of techniques, including physical, chemical, and biological ones. The biological technique, which employs biological elements including plants, microbes, and algae for the synthesis of NPs, is currently used as an alternate way of synthesis to get beyond these limitations. This approach has the advantages of being economical, somewhat toxic-free, and environmentally benign(Ghorbanpour *et al*., 2020).

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer and a leading cause of cancer-related deaths worldwide (Kim and Viatour, 2020). As a result, a full understanding of the biochemical foundations of this malignancy may lead to the development of fresh approaches to treatment (Alessandro *et al*., 2007).

According to several research, nanoparticles can interact with biological systems and pass through cell membranes because of their significant oxidative activity (Ndlovu *et al*., 2020). After crossing cell membranes, silver nanoparticles (Ag NPs) release their silver ions into the cell. These ions have negative effects on the biological system. They can cause genotoxicity, cyctotoxicity, and even cell death (Akter *et al*., 2017). Oxidative stress causes DNA and cell protein damage,

cell apoptosis, genetic defects, and even up regulation of the p53 gene. For these reasons, NPs may be used in medical applications as anticancer because up regulation of the p53 gene initiates apoptosis (Setyawati *et al*.,2013).

1.1.**Aim of this Study**

This study aims is to evaluate the antibacterial activity of crude leech salivary extract (CLSE), leech oil extract and leech salivary extract-mediated silver nanoparticles against some pathogenic bacteria and determination the cytotoxicity of Ag- NPs mediated leech saliva on HepG2 human cell line by the following steps:

1- Crude leech saliva extraction (CLSE) and leech oil extraction.
2- Evaluation antibacterial activity of (CLSE) and crude leech oil extract on test isolates ATCC bacteria(*E.coli, P. auroginosa, S. pyogenes and S. aureus)* by disc diffusion test.
3- Study the physical and chemical properties of LSE-AgNPs
4- Evaluation antibacterial activity of LSE-AgNPs mediated leech saliva on test isolates by agar well diffusion test, micro dilution assays and by MIC and MBC test.
5- Determination the cytotoxicity of LSE-AgNPs mediated leech saliva on HepG2 human cell line by MTT assay.

Chapter Two
Literature Review

2. Literature Review

2.1.Background of the Study

The segmented worm known as a leech (Hirudinea) belongs to the phylum Annelida (Yadav and Zhang, 2020). The Anglo-Saxon root "loece" (which meaning "to heal") is where the term "leech" originates. Egyptian artwork from 1500 BC show evidence of the use of leeches. It has been employed in medicine for "local depletion" (bloodletting) since ancient Greece, Rome, and Arabia (Hyson, 2005; Pourrahimi *et al*., 2020).

Based on their feeding habits, leeches are divided into two types. For instance, ferocious leeches prey on a variety of invertebrates. Another class of leeches are sanguivorous leeches, which are ectoparasites that feed on the blood of vertebrates, including people. With the aid of their suckers and biting jaws, leeches collect prey blood; after getting fully engorged, they drop off naturally without the need to continue feeding (Abdualkader *et al*., 2013). They can be found living both in damp terrestrial environments and in water (ponds, streams, lakes, and the ocean) (Shakouri and Wollina, 2021). Despite being widespread around the world, bloodsucking leeches are most prevalent in North America, Europe, and Southeast Asia (Ghosh, 2019).

Leech saliva is made up of a complex mixture of many physiologically active substrates that are necessary for the leech's survival and feeding activities in addition to possessing a variety of pharmacologically advantageous potentials (Ahirrao *et al*., 2017).

The high cost of pharmaceuticals in connection to poverty on the continent of Africa, the rate of antibiotic resistance, the toxic character of some chemically synthesized drugs, and the toxic nature of some chemically synthesized drugs have

all significantly contributed to the ineffectiveness on the treatment of infectious diseases (Chattu and Yaya, 2020). Now, efforts are focused on using natural resources to find effective, affordable, and safe medications to treat infectious diseases (Babay *et al*.,2022).

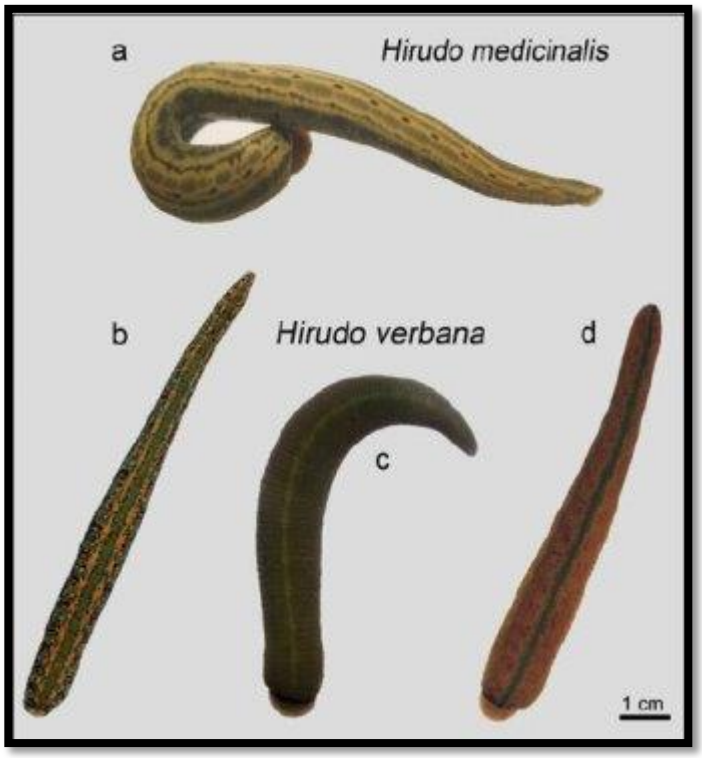
The newest and one of the most exciting fields of study in contemporary medicine is nanotechnology. When compared to larger particles of the bulk materials from which they are made, nanoparticles typically have new and improved properties based on size, distribution, and morphology (Albrecht *et al*.,2006; Pal *et al*.,2007; Bae *et al*.,2010; Iravani, 2014). Nanoparticles are typically a cluster of atoms that ranges in size from 1 to 100 nm.

In the fields of catalysis, optoelectronics, detection and diagnostic, antimicrobials and therapies, silver nanoparticles have a wide range of uses (Hong *et al*., 2006; Jain *et al*., 2009; Gottlieb and Nimmo, 2011; Sivakumar *et al*., 2012). It has been known that silver is a powerful antibacterial agent with low human toxicity and a variety of in vitro and in vivo uses (Farooqui *et al*., 2021).

2.2.Leech Morphological Characteristics

Leeches are invertebrates with a variety of colors, including brown, dark green, and black. They could have stripes on their bodies that are brown, orange, or red. A leech has suckers on the front and back. The posterior sucker is utilized for motility and attachment, whereas the anterior sucker is employed to draw blood. It has three jaws with a total of about 100 teeth each, and when it bites the skin, it leaves a distinctive Y incision. It uses its posterior sucker to crawl on land after swimming vertically and undulatingly in the water. It has two segmented tubular pumping hearts and no lungs; it breathes through its skin (Abdisa, 2018). Leech have no outer exoskeleton and a thin, flexible cuticle. Because it dries quickly, it is

closely related with water (Tilahun *et al*., 2020). Its mouth, which has three jaws arranged in a triradiate pattern, is located in the middle of the anterior sucker (Hyson, 2005). Leech bodies have 102 annuli, however their interior structures are divided into 32 segments (Porshinsky *et al*., 2011). According to (Ahirrao *et al*., 2017), the first four front segments are classified as head segments, the following 21 are midbody segments, and the final seven segments make up the tail sucker. Taxonomists can identify the genus and species of these worms by their sensory organs and annulation pattern figure (2.1).



Figure(2.1): (a) Adult living specimen of the European medicinal leech (A)*Hirudo medicinalis*, (b) *Hirudo verbena*, (c) dark pigmented form *H. verbana* " var. *nigra* " and (d) largely unpigmented variant *H. verbana* " var. *monostriata* ". (Kutschera, 2012)

2.3.Leech Locality and Ecology

Leeches may survive in a range of habitats, including moist terrestrial areas and aquatic situations. Freshwater, estuaries, rivers, ponds, lakes, and the ocean are all home to certain species. Leeches can endure the absence of water on moist terrain because some species have evolved with more mucous glands and larger nephridial vesicles (bladders) that retain and store extra water. Leeches also possess significant physiological flexibility, which allows them to adapt to a variety of environmental stresses like oxygen deficiency and temperature changes. Because moisture is a crucial element in the distribution and behavior of the terrestrial leech, it is common to find them in huge numbers in the woods and highlands of North America, Europe, and South East Asia. Leeches are active all year long in areas that are perennially humid, like Malaysia. while in regions with rainy and dry seasons, they go through an inactive and a dormant period (Yule and Yong, 2004).

2.4.The General Life Cycle of Leeches

The three life stages of leeches are egg, young leech, and adult. Most leeches mature in one to two years. An adult leech can survive for 18 to 27 years in a healthy environment and may go up to a year without eating if necessary. Eggs are laid between 1 and 9 months after copulation, at which point a specific organ called the "clitellum" starts to secrete four cocoons. There are roughly 15 eggs in each cocoon, yielding 60 offspring annually (Abdisa, 2018). Leeches are hermaphrodites, however in order to procreate, they require another leech (Porshinsky *et al*., 2011). Leeches have shown the ability to self-fertilize if suitable partners are not available (Phillips *et al*., 2020). After that, eggs are placed in moist soil and encased in a cocoon made of secretions that become polymeric when in

contact with water and the air. Young leeches emerge from their eggs and consume the liquid protein substance contained in the cocoon for the first few days of their existence. After emerging from the cocoon, leeches mostly consume the bodily fluids of amphibians since their skin is sensitive and thin and may be easily pierced by the fangs of juvenile leeches (Elliott, 2008) figure (2.2).

Figure(2.2): (A) Adult and juvenile alcohol-preserved *H. medicinalis*, and a cocoon in dorsal view. (B) The Inset shows the characteristic pigment pattern of a newly hatched individual in dorsal and ventral view, respectively. (Kutschera and Roth, 2006; Fernadez and Matte 2010) .

2.5.Leech Physiological Functions

Leeches may identify suitable environments and approaching prey or hosts by using a range of sensory structures. The most distinctive are photoreceptors (the eyes), chemoreceptors (for chemicals in the air and water), and mechanoreceptors (for vibrations or sounds). Leeches use all three senses to find potential prey. Photoreceptors are unable to create high-resolution images, although they can detect light and dark as well as some movement (Phillips *et al*., 2020).

Leech is sensitive to a wide range of substances, light, touch, sound, and water waves. Through repeated contractions, they can bite and sucking the blood.

A leech consumes 10-15 ml of blood at a time and digests it in an average of 40 minutes (Shakouri and Wollina, 2021). Since a leech only consumes, On rare circumstances, a leech can consume more than ten times its own body weight in blood from a suitable host in a single blood meal (Lent *et al*., 1988). In order to preserve it, the blood is collected in a crop with ten pairs of diverticula. In the host's plasma, more salt and water are ejected, and the concentrated material is stored in the crop for several months (Zerbst-Boroffka and Wenning, 1986; Wenning, 1996). *Aeromonas spp*., symbiotic bacteria found in leeches, are present in the crop and may delay spontaneous or microbial food deterioration (Graf *et al*., 2006).

Crop material is delivered to the intestine in little amounts and digested on a regular basis (Roters and Zebe, 1992). Inedible Leech is released from the anus; threat, intervention, a pharmacy, etc. 119 elements, chiefly derivatives of hem. The leech starts moving its jaws while also releasing saliva. Over the course of the entire feeding cycle, the leech may empty the gland reservoirs (Hildebrandt and Lemke, 2011).

2.6.The Biology of Leech Feeding

Both sanguivorous and predatory leeches use their intestines to digest their food. Only months' worth of blood are stored inside the bodies of sanguivorous creatures. Actually, hematophagous leeches go through a number of sluggish stages in the digestion of blood, allowing them to retain the ingested blood for up to 18 months. In the leech's gut, there are symbiotic bacteria known as *Aeromonas* spp., including Rikinella-like species, *Aeromonas veronii*, and *A. hydrophila*. According to various studies (Indergand and Graf, 2000; Siddall, *et al*., 2011; Bomar, *et al*., 2011; Maltz and Graf, 2011; Maltz *et al*., 2014), symbionts like *A.*

veronii secrete enzymes that aid in both the breakdown of the ingested blood's constituent parts and the production of antibiotics to stop blood putrefaction. Another alleged function avoid B complex deficiency, which frequently develops in animals that depend on blood nourishment (Sawyer,1986; Yule and Yong 2004).

2.7.Historical Review of Leeching

Both conventional and modern medicine rely heavily on medicinal leeches (Whitaker *et al*., 2004; Elliott and Kutschera, 2011). Around 1500 BC, a painting of an Egyptian tomb depicted leeches being employed for therapeutic purposes. This is the first legibly recorded instance of such use. In Europe, the popularity of hirudotherapy peaked in the 17th and 18th century AD, but in the Arab world, leeches were only employed for bloodletting (Munshi *et al*.,2008).

However, Nicander of Colophon's writings from the first century BC have survived to the present day (Papavramidou and Christopoulou-Aletra, 2009). Leech therapy has been practiced for centuries by Unani doctors who follow Galen's (130–201 AD) hypothesis, which was influenced by Hippocrates (460–370 BC), that ailments in the body are brought on by humeral imbalance. In his work Canon of Medicine, Avicenna also advocated leech therapy, particularly for skin conditions (Lone *et al*., 2011).

When two Slovenian surgeons utilized the parasites to help with circulation following tissue-flap transplantation, leeching was first used in modern microvascular surgery and tissue transfer. Leeches were employed in the post-operative management of a scalp avulsion case, according to a 1983 report by Henderson *et al*. A five-year-old boy's ear was successfully reattached in 1985 by Harvard doctor Joseph Upon using medicinal leeches (Mutimer *et al*.,1987).

Since then, leeches have been widely used to salvage vascularly compromised flaps, or muscle, skin, and fat tissue surgically transferred from one part of the body to another, and replants, limbs, or other body parts reattached, as well as to reduce venous congestion in fingers, toes, ears, and scalp reattachments. Amputation due to trauma. Leeches of the *Hirudo medicinalis* species were in risk of going extinct due to the overuse of this substance in Europe. As a result, laws were developed to govern the leech trade. Therefore, when leech populations declined and alternative therapeutic options emerged, demand for leech therapy fell (Munshi *et al*., 2008).

The United States Food and Drug Adminstration clearance in 2004 to utilize *Hirudo medicinalis* for therapeutic purposes in plastic and reconstructive surgery and subsequent study on the composition of leech saliva rekindled interest in hirudotherapy (Mumcuoglu, 2014).

Despite being employed in traditional medicine since ancient times, medicinal leeches are now one of the alternative treatment methods of modern medicine thanks to scientific evidence of the efficacy of the active chemicals they secrete (Sig *et al*., 2017).

2.8.*Hirudo medicinalis*

According to Davis and Appel (1979) and Solijonov and Umarov (2022), the medicinal leech (*Hirudo medicinalis*) is a segmented annelid that is a member of the Phylum Annelida, which is a large phylum that contains 22.000 species. This group of animals has significant impacts on the environment, agriculture and health. Class : Clitellata, and Subclass: Hirudinea. Hematophagous animals, which consume the blood of their prey, it was discovered that the substances which are present in leech saliva play a great role in preventing thrombosis of blood and

improving blood circulation have been observed to prevent blood clotting by secreting a variety of physiologically active substances, including the anticoagulants, in their salivary gland secretions (Babenko *et al*., 2020).

Therapists use leech therapy for a variety of ailments for decades since leeches first caught their notice. The majority of doctors favored the European medical leech species, *Hirudo medicinalis*, figure(2.3) popularly known as the healing leech, for various therapeutic uses, as opposed to *Hirudo decora*, an American species, can suck less blood since it just makes a small, superficial cut in its prey's skin (Whitaker *et al*., 2004). Many other species, including *Hirudinaria manillensis* (Electricwala *et al*., 1991), *Hirudo nipponia* (Kim and Kang, 1998), *Hirudo verbena*, *Hirudo orientalis* (Baskova *et al*., 2008), and *Haementeria depressa*, were also thought to be useful as medical tools.

Figure (2.3) *Hirudo medicinalis* (Wikipedia, 2023)

2.9. Scientific Classification of Leech (*Hirudo Medicinalis*)

Domain: Eukarya

Kingdom: Animalia

Phylum: Annelidia

Class: Clitellata

Order: Arhynchobdellida

Family: Hirudidae

Genus: Hirudo

Species: *Hirudo medicinalis*

(Wikipedia, 2021)

2.10.Modern Applications of Leeching

2.10.1.Infectious Diseases

As a result of the commercially accessible antibiotics being used more frequently due to the prevalence of infectious diseases, a new and difficult phenomena known as antimicrobial agent resistance has emerged as a result, researchers have established new plans to create antibiotics with new mode of action and decreased rates of bacterial resistance (Tasiemski and Salzet, 2012).

Numerous reviewers who looked into the therapeutic value of the medicinal leech noted that leeching might be successful in treating infections without providing any further information or explanation on leech application procedures and the make-up of the active ingredient. For instance, some claim that traditional

dentists have treated dental illnesses such periodontitis and alveolar abscesses with leech therapy (Srivastava and Sharma, 2010).

From the therapeutic leech extract, a protein called destabilize with lysozyme-like activity had been identified. Because it can destroy the cellular components of some bacterial strains, it has been claimed that this protein has antibacterial action (Zavalova *et al*., 2000; Zavalova *et al*., 2006). Additionally, it was noted that the European leech, *H. medicinalis*, nervous system was capable of signaling the synthesis of AMPs in order to start an antimicrobial response in the event of injury(Schikorski *et al*., 2008).

From this type of leech, three distinct peptides with antibacterial properties were discovered. From neurons and microglial cells, *H.m. lumbricin* and neuromacin were extracted, while (Tasiemski *et al*., 2008) Leech bodily fluids included peptide B. The use of leech extract from numerous leech species in the family Hirudinidae as an antibacterial agent with varied applications was recently patented by certain researchers. The pure extract from any area of the leech body, particularly the salivary glands, they said, had antibacterial action against a variety of Gram negative and positive infections. They claimed that *Shewanella* and *Aerococcus viridans* were very resistant to leech extract's antibacterial effects, while *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* showed less effects. They explained how leech extract could be utilized to treat diseases caused by bacteria, such as nosocomial infections, foodborne illnesses, and arthritis (Tasiemski and Salzet, 2012).

Last but not least, saliva produced by the tropical leech *H. manillensis* was found in leech body fluids (Tasiemski *et al*.,2008). Recently, some researchers patented the usage of the leech extract from many leech species of the family

Hirudinidae as an antimicrobial agent with various applications. They argued that the purified extract obtained from any part of leech body, especially salivary glands, showed an antimicrobial activity against many Gram-negative/positive pathogens. Finally, it was shown that the salivary gland secretion from the tropical leech *H. manillensis* had broad-spectrum antibacterial activity against bacterial strains that are both Gram positive *S. aureus* and Gram negative *S. typhi and E. coli* (Abdualkader *et al*.,2011).

2.10.2. Cancer and Metastasis

In 2008, 13% of all fatalities worldwide were attributable to cancer. These frightening rates are anticipated to rise over the following 20 years, reaching 13.2 million deaths by 2030 (Jemal *et al*., 2011). The fact that leech therapy has not been proven to be a cytotoxic agent for the treatment of cancer in scientific studies was taken into consideration when conducting this review. The review was conducted based on several studies that were focused on employing leech extract and saliva as antimetastatic agents rather than using them to treat the tumor directly (Abdualkader *et al*., 2013).

A previously discovered metastatic inhibitory action of various anticoagulants, such as warfarin and heparin, served as the inspiration for the use of leeches as an antimetastatic agent (Wallis *et al*.,1992). It was thought that leech saliva would be a more potent antimetastatic medication due to its very high concentration of anticoagulants, protease inhibitors, and other substances (Gasic, 1986). Later, the salivary gland secretion of the proboscis leech, *H. ghilianii*, was isolated to produce the antimetastatic and anticoagulant protein known as ghilanten (Blankenship *et al*.,1990). Ghilanten was said to be able to prevent the spread of melanoma, breast cancer, lung cancer, and prostate cancer (Cardin *et al*., 1994). An

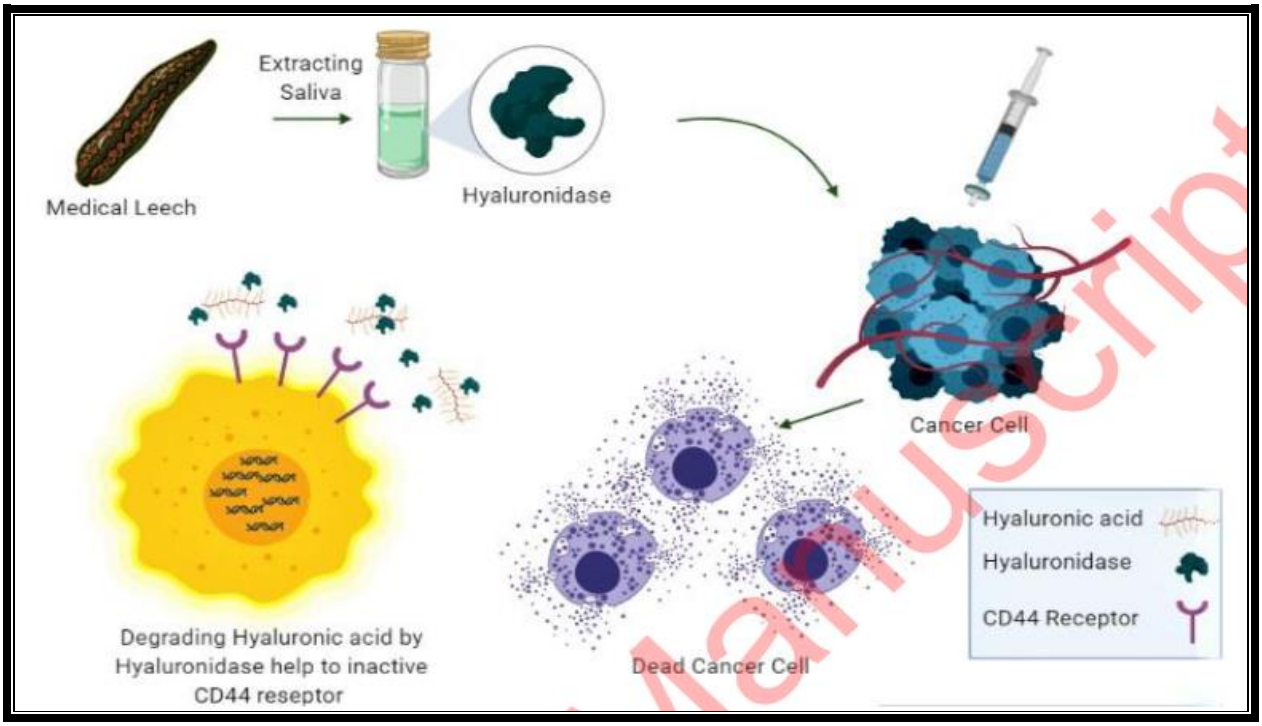
effective metastasis inhibitor of a variety of malignant tumor cells, including lung carcinoma, breast carcinoma, bladder carcinoma, colorectal carcinoma, soft-tissue sarcoma, leukemia, and lymphoma, was described by another study using a synthetic hirudin preparation(Wallis *et al*.,1992).

Saliva from the Mexican leech *Haementeria officinalis*, possesses antimetastatic qualities. Lung cancer is prevented from colonizing by the protein antistasin, which is present in its saliva. The secretions contain antiproteolytic enzymes, anticoagulants, and platelet aggregation inhibitors. *H. manillensis*, a distinct tropical leech, has demonstrated an antiproliferative impact *in vitro* when used to treat small cell lung cancer (Abdualkader *et al*., 2013)

Researchers revealed for the first time that patients with late stages of kidney cancer and leiomyosarcoma might receive a 2-month treatment by topical application of *H. medicinalis* and be entirely cured of their local lumbar pain (Kalender *et al*., 2010). A saliva extract from the tropical leech *H. manillensis* was recently shown to have antiproliferative properties against small cell lung cancer (SW1271). Additionally, the leech saliva demonstrated supra-additive synergistic action with carboplatin (Merzouk *et al*., 2012).

Based on achieving blood circulation across the flap, a patient with basal cell carcinoma underwent leech therapy for 9 months after surgery and experienced good results. In a patient with intraoral cancer, the medical leeches are successful in reducing venous obstruction of a free forearm flap followed by reconstructive surgery. The salivary gland secretions of the leech have antimetastatic properties and contain enzymes that are antiproteolytic, decrease platelet aggregation, that prevent clotting. The saliva of the leech contains a protein known as antistasin, which prevents lung cancer from colonizing. Furthermore, other components like

hyaluronidase include anti-tumor action. According to theory, hyaluronidase anticancer activity may occur to some extent by inhibiting pro-tumorigenic immune cells into the tumor stroma by destroying the hyaluronic acid-CD44 interface (Ammar *et al*., 2015), figure(2.4).



Figure(2.4): Activity of Hyaluronidase in Cancer. Degrading hyaluronic acid by hyaluronidase, inhibiting CD44 activation and after a while mRNA of VEGF reduced and it helps to reducing angiogenesis of cancer cells (Shakouri and Wollina, 2021).

2.10.3.Pain Management

Leeches are used to treat several types of pain disorders. Sometimes the pain relief is immediate, other times it takes a while. According to reports, leech therapy may help with very bad cancer pain. Studies on osteoarthritis have suggested that leech therapy can improve symptoms by having an anti-inflammatory and analgesic effect (Wollina *et al*.,2016).

In a case series evaluating the efficacy of leech therapy, four patients with varicose ulcers were included in this study. Medical leech therapy was applied to four patients of different ages, being affected by different diseases and who had ulcers on their legs. In this case series, out of four cases, three cases of the ulcers were completely healed and also showed remarkable improvement in other variables such as pain, discomfort on walking and itching (Ünal *et al*., 2023).

Some numbers of reports show the effectiveness of leech therapy on the salvage of organs damaged by traumatic injuries. Leech therapy was applied to the patient with a laceration of the right ear after a vehicle accident to reduce venous congestion in this region. At the early stage of wound healing, the ear exhibited marked improvement, and signs of adequate revascularization began to appear in this area after the medicinal leech therapy. At the late stage of wound healing, the ear exhibited signs of complete revascularization (Ünal *et al*., 2023).

2.11. Safety and Complication of Leeching

Leeching's most frequent side effect, infection, affects between 2 and 36% of patients (Green and Shafritz, 2010). The agent is the Gram-positive rod *Aeromonas hydrophila,* which can cause pneumonia, muscular necrosis, flap failure, and even septicemia. These infections involve *Aeromonas* spp., *Pseudomonas* spp., and *Vibrio* spp. Aminoglycosides and fluoroquinolones should be included in the treatment regimen for such infections because *A. hydrophila* are resistant to penicillins and the first generation of cephalosporin (Srivastava and Sharma, 2010; Porshinsky *et al*.,2011) However, despite the fact that doctors who perform leeching are encouraged to use a leech once (Michalsen *et al*., 2007), there are no records of infections being transferred through leech therapy.

Numerous studies have described local hypersensitivity problems that may be brought on by the presence of certain toxins in leech saliva, including itching, blister formation, ulcerative necrosis, and even local tissue destruction (flap death)(Srivastava and Sharma, 2010). Post leeching consequences have also been described as blood loss due to prolonged hemorrhage and skin marks (scars) caused by delayed healing of leech bites (Green and Shafritz, 2010).

2.12.Leech Works With Secreted Proteins

The impact mechanisms of leeches have been the subject of numerous scientific research to date. Only a small number of the more than 100 distinct proteins with various molecular weights that have been found in leech secretions have been recognized as playing a significant active role (Baskova *et al*., 2004), figure (2.5).

The first bite of leech therapy is followed by a bonding period of 20 to 45 minutes during which the leech feeds on blood. It was often thought that leeches feeding on their hosts' blood provided the primary therapeutic advantages, but research has since demonstrated that the benefits were really brought about by the bioactive compounds present in the leech saliva that were discharged into the host's bloodstream during sucking (Abdullah *et al*.,2012**).** Table(2.1) lists the bioactive substances present in leech saliva along with their intended uses. To make the effect mechanisms more understandable, they are separated into six sorts. However, because these mechanisms are interconnected, they should be assessed collectively. After biting, a leech must create a pathway for sucking (extracellular matrix degradation), prevent adhesion, aggregation, and coagulation (inhibition of platelet functions, and anticoagulant effect), increase blood flow, defend itself

(antimicrobial activity), and hide from detection (analgesic and anti-inflammatory effects)(Montinari and Minelli, 2022).

Histochemical analyses showed the lack of digestive enzymes such endopeptidases, lipases, or glucolytic enzymes in the gastrodermis, the epithelium lining the leech's digestive tract. The gastrodermis is not morphologically differentiated into secretive and absorptive parts (Jennings & van der Lande 1967; Sawyer 1986).

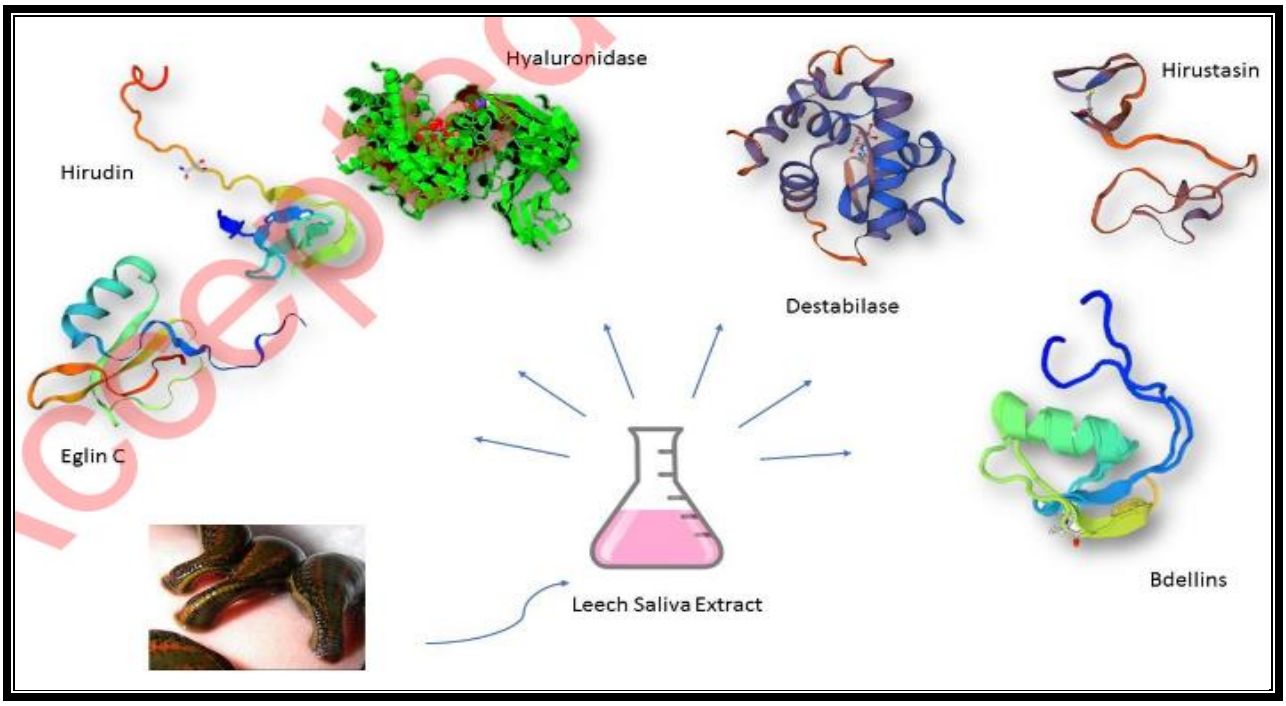
Conversely, it was discovered that exopeptidases and basic and acidic phosphatases were extremely active (Jennings & van der Lande 1967; Fischer 1970; van der Lande 1972). The majority of leeches' food digestion and absorption processes, according to the quoted authors, take place in their intestines, where exopeptidases (arylamidases) are the only enzymes that can break down proteins. Leeches take longer to digest proteins because they lack endopeptidases, which in most other animals start the digestive process. For *H. medicinalis*, however, different outcomes were seen (Zebe *et al*. 1986; Roters & Zebe 1992b).

Three endopeptidases from the serine protease class were found to be active in the gut of this species. Since these enzymes differ greatly from the serine proteases present in vertebrates, it is most likely the leech that produces them. Trypsin-like proteases make up one of them, while chymotrypsin-like proteases make up the other two. In the intestine of this species activities of three endopeptidases belonging to the class of serine proteases were detected. These enzymes are most probably produced by the leech, as they significantly differ from serine proteases found in vertebrates. One of them is a trypsin-like protease, the other two are chymotrypsin-like ones. (Zebe *et al*. 1986; Roters & Zebe 1992a; Baskova & Zavalova 2001)

Table (2.1) The Bioactive Compounds Found in Leech Saliva and Their Functions(Baskova and Zavalova 2001; Zaidi *et al*.,2011; Sig *et al*.,2017).

Modes of Action	Substance	Target or Function
Analgesic and Anti-İnflammatory Effects	Bdellins	Inhibits trypsin, plasmin, and sperm acrosin
	Hirustasin	Inhibits tissue (but not plasma) kallikrein
	LDTI (leech-derived tryptase inhibitor)	Inhibits tryptase
	Eglins	Inhibits α-chymotrypsin, chymase, subtilisin, and the neutrophil proteinases elastase and cathepsin GInhibitor.
	LCI (leech carboxypeptidase)	Inhibits Carboxypeptıdase A
	Complement C1 Inhibitor	It can bind to complement-fixing sites of antibodies (IgG and IgM)
	Guamerin from Hirudo nipponia	Inhibits Leukocyte-elastase Specifically
	Piguamerin from Hirudo nipponia	Inhibits kallikrien, and trypsin
Anticoagulant Effects	Hirudin	Inhibits thrombin
	Factor Xa Inhibitor	Inhibits Factor Xa
	Destabilase	Dissolves stabilized fibrin
	Gelin	Inhibits elastase, cathepsin G, and chymotrypsin
Extracellular Matrix Degradation	Hyaluronidase	Targets endoglucoronidic linkages of hyaluronic acid
	Collagenase	Dissolves the collagen particles
Anti-Platelet Effects	Apyrase	Targets Adenosine 5' Diphosphate, arachidonic acid, platelet-activating factor (PAF), and epinephrine
	Calin	Inhibits collagen-induced platelet aggregation (directly) or von- Willebrand factor collagen binding (indirectly)
	Saratin	Inhibits the binding of α2 integrin subunit I domain to collagen
The Effects on Blood Flow	Acetylcholine	Targets blood vessels
	Histamine-like Substances	Targets blood vessels
Antimicrobial	Destabilase	The β1–4 bonds of the peptidoglycan layer in

Effects		the bacterial cell wall
	Chloromycetin	Bacterial protein synthesis
	Theromacin	The bacterial membrane
	Theromyzin and Peptide B	



Figure(2.5): Medical leech saliva contains wide range of proteins and enzymes which have multifunctional effect in diseases (Shakouri and Wollina, 2021).

2.13. Future Prospects of Leech Therapy

Leech therapy has a long history but has experienced periods of popularity and acceptance before falling out of favor. Leech Therapy can be learned very quickly compared to other complementary therapies and natural treatments, which helps to lessen the difficulties brought on by the unneeded use of synthetic medications. Today, research is being done to determine the therapeutic effect of leeches in many disease situations, such as male and female sterility, diabetes,

lupus erythromatosis, and prostate problems and many others (Shakouri and Wollina, 2021).

2.14.Bacterial Isolates

2.14.1.*Escherichia coli*

E. coli is a gram-negative, non-sporulating, rod-shaped, facultative anaerobic, and coliform bacterium pertaining to the genus *Escherichia* that commonly inhabits the environment, foods, and warm-blooded animals' lower gut (Robbens *et al*., 2014). It can live for long periods of time in feces, soil, and water, and is frequently used as a water contamination indicator organism. Cells are typically rod-shaped, It is motile due to peritrichous flagellar arrangement, and very few strains are non-motile. The optimal growth of *E. coli* occurs at 37°C. Fimbriated strains exist both as motile and non-motile. A polysaccharide capsule has been discovered in some *E. coli* strains isolated from extra intestinal infections. The *E. coli* capsules can be clearly seen using negative staining procedures, which produce a bright halo over a dark backdrop. They have a thin cell wall with only one or two layers of peptidoglycan (Köhler and Dobrindt, 2011).

Some serotypes can induce diarrhea when consumed through contaminated food or drink, while others might cause urinary tract infections (UTIs), anemia, and respiratory or kidney infections. In order to cause disease *E. coli* to possess several different types of virulence factors: fimbrial and fimbrial adhesins, capsules, toxins (exotoxins, hemolysins, and enterotoxins), iron up-take systems, etc (Robbens *et al*., 2014).

E. coli cells may grow on a solid or in a liquid growth medium under laboratory conditions. It may be grown in a basic minimum of media, which

includes glucose as a carbon and energy source, ammonium salts as a nitrogen source, other salts, and trace elements (Elbing and Brent, 2019). As *E. coli* have simple nutritional requirements it can be easily cultured on a common medium, such as Nutrient agar, MacConkey agar, and EMB agar and blood agar (Elbing and Brent, 2019). *E. coli* can grow at temperatures ranging from 10°C to 40°C, although the optimum temperature for most strains is 37°C (Robbens *et al*., 2014).

2.14.2. *Pseudomonas aeruginosa*

P. aeruginosa is a common encapsulated, Gram-negative, aerobic–facultatively anaerobic, rod-shaped bacterium that can cause disease in plants and animals, including humans (Wood *et al*., 2022). A species of considerable medical importance, *P. aeruginosa* is a multidrug resistant pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses – hospital-acquired infections such as ventilator-associated pneumonia and various sepsis syndromes(Diggle and Whiteley, 2020).

The organism is considered opportunistic insofar as serious infection often occurs during existing diseases or conditions – most notably cystic fibrosis and traumatic burns. It generally affects the immunocompromised but can also infect the immunocompetent as in hot tub folliculitis. Treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics. When more advanced antibiotic drug regimens are needed adverse effects may result (Høiby *et al*., 2010).

It is citrate, catalase, and oxidase positive. It is found in soil, water, skin flora, and most human-made environments throughout the world. It thrives not only in normal atmospheres, but also in low-oxygen atmospheres, thus has colonized many natural and artificial environments. It uses a wide range of organic material for

food; in animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonization occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (Wood *et al*., 2022). Because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills *P. aeruginosa* is capable of extensive colonization, and can aggregate into enduring biofilms (Diggle and Whiteley, 2020).

P. aeruginosa produces colonies with a characteristic "grape-like" or "fresh-tortilla" odor on bacteriological media. In mixed cultures, it can be isolated as clear colonies on MacConkey agar (as it does not ferment lactose) which will test positive for oxidase. Confirmatory tests include production of the blue-green pigment pyocyanin on cetrimide agar and growth at 42 °C. A TSI slant is often used to distinguish nonfermenting *Pseudomonas* species from enteric pathogens in faecal specimens (Diggle and Whiteley, 2020).

2.14.3.*Staphylococcus aureus*

S. aureus is a major bacterial human pathogen that causes a wide variety of clinical manifestations (Raineri *et al*., 2022). *S. aureus* is Gram-positive bacteria (stain purple by Gram stain) that are cocci-shaped and tend to be arranged in clusters that are described as “grape-like.” On media, these organisms can grow in up to 10% salt, and colonies are often golden or yellow (aureus means golden or yellow). These organisms can grow aerobically or anaerobically (facultative) and at temperatures between 18 C and 40 C. Typical biochemical identification tests include catalase positive (all pathogenic *Staphylococcus* species), coagulase

positive (to distinguish *S. aureus* from other *Staphylococcus* species), novobiocin sensitive (to distinguish from *S. saprophyticus*), and mannitol fermentation positive (to distinguish from *S. epidermidis*) (Rasigade and Vandenesch, 2014).

Staphylococci grow readily on most bacteriologic media under aerobic or microaerophilic conditions. Colonies on solid media are round, smooth, raised, and glistening. *S. aureus* usually forms gray to deep golden yellow colonies. Mannitol Salt Agar: circular, 2–3 mm in diameter, with a smooth, shiny surface; colonies appear opaque and are often pigmented golden yellow. Tryptic Soy Agar: circular, convex, and entire margin. Blood Agar: beta-hemolysis. Brain heart infusion agar: Yellow pigmented colonies (Raineri *et al*., 2022).

2.14.4.*Sterptococcus pyogenes*

The group A β-hemolytic streptococci are gram-positive cocci and produce clear zones of hemolysis (β) on blood agar, differentiating them from streptococci producing partial (α) and non-hemolytic (γ) streptococci.1 *S. pyogenes* is the only organism within group A *streptococcus* (GAS). Group A *streptococcus* transmission is via droplets from pharyngeal infection or colonization, direct contact, contaminated fomites, or foodborne contamination. Group A *streptococcus* can cause a broad Invasive GAS diseases are generally defined as clinical diseases associated with the isolation of *S. pyogenes* in sterile areas such as blood, cerebrospinal or pleural fluids, deep wounds, and muscles (Shahin *et al*., 2022).

Streptococci are generally grown on agar media supplemented with blood. This technique allows the detection of β-hemolysis, which is important for subsequent identification steps, and enhances the growth of *streptococci* by the addition of an external source of catalase. Selective media for culturing Gram-

positive bacteria (such as agar media that contains phenylethyl alcohol, or Columbia agar with colistin and nalidixic acid) also provide adequate culturing conditions for *S. pyogenes*. Optimal incubation conditions for the vast majority of streptococcal strains include a temperature range of 35°C to 37°C in the presence of 5% CO2 or under anaerobic conditions (Shahin et al., 2022).

To identify *S. pyogenes* in clinical samples, blood agar plates are screened for the presence of β-hemolytic colonies. The typical appearance of *S. pyogenes* colonies is dome-shaped with a smooth or moist surface and clear margins. They display a white-greyish color and have a diameter of > 0.5 mm, and are surrounded by a zone of β-hemolysis that is often two to four times as large as the colony diameter. Microscopically, *S. pyogenes* appears as Gram-positive cocci, arranged in chains (Atomsa, 2022).

2.15.Antibiotics and Mechanisms of Action

2.15.1. Antibiotics

Antibiotic is a specific metabolic product produced by a microorganism or modifier (produced wholly or partly by chemical synthesis) which, in low concentration, inhibits the growth of other microorganisms. The most important concept underlying antimicrobial therapy is selective toxicity. Source of antibiotics There are three major sources from which antibiotics are obtain; Microorganisms: most of antibiotics are produced by fungi (eg., *Penicillium*, *Cephalosporium*), or by bacteria (eg., *Streptomyces*). Synthesis: eg., chloramphenicol, sulphonamides. Semisynthetic: this means that part of the molecule is produced by a fermentation process using the microorganism and the product is then further modified by a chemical process eg., ampicillin(Uddin *et al*., 2021).

2.15.2.1Ampicillin

Ampicillin is in a group of medications known as penicillins. Ampicillin is classified under aminopenicillins (broad spectrum penicillins). Ampicillin is a semi-synthetic derivative of penicillin that functions as an orally active broad spectrum antibiotic (Bereda *et al*., 2022).

2.15.2.2. Mechanism of Actions

Ampicillin prevents the final transpeptidation step of peptidoglycan synthesis in bacterial cell wall by binding to one or more of the penicillin-binding proteins, thus preventing cell wall biosynthesis resulting in bacterial lysis; Ampicillin exerts bactericidal action on both gram positive and gram-negative organisms. Its spectrum involves gram positive organisms such as, example *Streptococcus pneumoniae* and other *streptococci*, *Listeria monocytogenes*, and gram negative, example *Moraxella catarrhalis*, *Neisseria meningitidis*, *Escheria coli*, *Salmonella* (Baranowski *et al*., 2018)

Ampicillin exerts its action by preventing the synthesis of bacterial cell wall or ampicillin prevents bacterial cell wall synthesis by binding to penicillin binding proteins (PBPs), which are the enzymes accountable for the formation of the cell wall structure (Bereda, 2022).

Ampicillin, like all penicillins, acts as a structural analogue of acyl-D-alanyl-D alanine and acylates the transpeptidase enzyme responsible for the final stage of the formation of the peptidoglycan, which is the chief component of the cell wall (Bereda, 2022).

2.15.3.1.Amikacin

Amikacin's niche is because it also has activity against more resistant gram-negative bacilli such as *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. It also has excellent activity against most aerobic gram-negative bacilli from the *Enterobacteriaceae* family, including *Nocardia sp*. and some *Mycobacterium spp*. (*M. avium-intracellulare, M. chelonae, and M. fortuitum*). Unlike gentamicin, amikacin does not provide synergistic activity against *Enterococcus faecium* when combined with beta-lactam antibiotics (Ramirez and Tolmasky, 2017).

2.15.3.2. Mechanism of Action

Amikacin binds to the 30 S bacterial ribosome subunit, resulting in interference with a reading of the genetic code and inhibition of protein synthesis, e.g., elicits premature protein termination and incorporation of incorrect amino acid. Amikacin, as well as the rest of the aminoglycosides, are generally bacteriocidal and probably have an additional mechanism of action, which as yet remains undetermined. Aminoglycosides demonstrate bacterial killing that is concentration-dependent and also have a post-antibiotic effect (Block and Blanchard, 2022)

Amikacin, when combined with penicillins, can have an additive effect on specific microorganisms. Amikacin, when combined with carbapenems, can have a synergistic effect against some gram-positive organisms. Amikacin may retain activity against tobramycin- and gentamicin-resistant strains because of reduced inactivation by bacterial acetylase, adenylase, and phosphorylase. Thus, its routine clinical use should be reserved for difficult to treat serious nosocomial infections (Block and Blanchard, 2022).

2.15.4.1 Ceftriaxone

Ceftriaxone is a broad-spectrum β-lactam (cephalosporin/cephamycin) antibiotic that displays in vitro activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria. The bactericidal activity of ceftriaxone results from inhibition of cell wall synthesis and is mediated through ceftriaxone binding to penicillin-binding proteins. Ceftriaxone is stable against hydrolysis by a variety of β-lactamases including penicillinases, cephalosporinases, and extended-spectrum β-lactamases (Bush and Bradford, 2016).

2.15.4.2. Mechanism of Action

Ceftriaxone works by inhibiting the mucopeptide synthesis in the bacterial cell wall. The β-lactam core of ceftriaxone binds to carboxypeptidases, endopeptidases, and transpeptidases in the bacterial cytoplasmic membrane. These enzymes are involved in cell wall synthesis and cell division. By binding to these enzymes, ceftriaxone causes formation of defective cell walls and promotes cell death. Ceftriaxone is often used in combination with macrolide and aminoglycoside antibiotics for the treatment of pneumonia. It is also a choice drug for treatment of bacterial meningitis. In pediatrics, it is commonly used in febrile infants between 4 and 8 weeks of age who are admitted to hospital in order to exclude sepsis. Ceftriaxone has also been used to treat Lyme disease, typhoid fever, and gonorrhea (Bush and Bradford, 2016).

2.16.Silver Nanoparticles and Their Application as an Antibacterial Agent

According to (Kesharwani *et al*., 2018), nanotechnology is the design, development, and application of structures, devices, and systems by manipulating

shape and size at a nanometer scale (1 nm to 100 nm). It is a novel area of study with many scientific and technological uses, especially for creating new materials. Nanoparticles are generated with special features that make them valuable in biology and materials science. Silver nanoparticles have become one of the most studied types of nanoparticles in recent years (Saravanan *et al*.,2018).

Silver nanoparticles typically have sizes of less than 100 nm and contain 20 to 15,000 silver atoms. Silver nanoparticles have exceptional antibacterial activity even at low concentrations because of their high surface-to-volume ratio (Oves *et al*., 2018). Additionally, they have low cytotoxicity and little immunological reaction, and they are inexpensive (Samuel *et al*., 2020). As a result, there are numerous potential biomedical uses for silver nanoparticles. According to (Pugazhendhi *et al*. , 2018), they are employed in molecular diagnostics, medical imaging, and drug delivery. Additionally, they are utilized in therapeutic products including surgical mesh, the creation of artificial joint replacements, wound dressing, and medications that speed up the healing of wounds (Shanmuganathan *et al*.,2019).

Particles with a size between 1 and 100 nanometers (nm) are known as nanoparticles (NPs). The creation of nanoparticles has involved a wide range of techniques, including chemical, physical, biological, and biogenic ones (Patra and Baek, 2014). The production of equivalent metal NPs through biogenic reduction of metal precursors, on the other hand, has shown out to be more cost-effective, chemically free, and environmentally benign. For the biogenic reduction of natural materials like protein-rich plant extracts or animal secretions that are also embedded with natural stabilizing, growth-inhibiting, and capping compounds have proven an excellent supply for metallic particles to nanoparticles. A few pathogenic species have been shown to be resistant to metallic nanoparticles made

through biogenic reduction, such as silver nanoparticles (Ag-NPs). Due to their special qualities, structures, and sizes, they are also used in biomedical sciences for bio-imaging, medication delivery, cancer treatment, medical diagnosis, and sensor fabrication. Silver nanoparticles with tiny sizes are created when silver nitrate and leech saliva are combined. These particles can operate as a drug carrier by absorbing bioactive molecules into their matrix, which may limit the growth of infections (Nadaroglu et al., 2017).

2.17.Green Synthesis of Silver Nanoparticles

The manufacture of nanoparticles using non-hazardous techniques or non-chemical reagents is referred to as "green synthesis" of nanoparticles. Reduced environmental toxicity and health risks are the main goals of this technology (Iravani *et al*., 2014; Rafique *et al*., 2017). To solve this issue, researchers developed precise routes that can be utilized to synthesize nanoparticles from naturally existing materials and their byproducts (Anastas and Eghbali, 2010; Tejamaya *et al*., 2012).

The use of biological mechanisms to synthesize nanoparticles makes them very valuable. Scientists laid the groundwork for future green synthesis in the 19th century by figuring out how to reduce biological materials (Kumar *et al*., 2016). The reduction of Ag+ to Ag0 is a key step in the majority of green silver nanostructure manufacturing techniques, carried out by biological species or by biologically derived substances of the relevant class of plant or creature, performed by biological species or by compounds of biological origin of the appropriate type of plant or organism (Sharma *et al*.,2009; Iravani *et al*.,2014).

Compared to physical and chemical approaches, biological approaches provide a number of benefits. In contrast to chemical treatments, they are firstly

more environmentally friendly (Srikar *et al*., 2016). They are energy efficient since they use less energy than physical processes (Iravani *et al*., 2014). They are utilized for mass production because they are economically viable (Iravani, 2011). They are a method because their regenerative nature makes a wide range of chemicals available that can function as reducing agents (Naghdi *et al*., 2015).

Along with the above mentioned benefits, the procedure is extremely effective. The optimization of the method in terms of scalability, product quality, and efficiency is crucial for the green synthesis of Ag-NPs. By altering the pH, temperature, redox conditions, incubation time, and salt content, the reaction conditions can be made better. (Jorge de Souza *et al*., 2019).

For instance, pH changes in plants affect the charge of phytochemicals, which alters the reduction and binding of Ag throughout the synthesis process (Iravani, 2011; Singh, 2016). pH fluctuations can also affect the size of nanoparticles. The choice of an effective technique for removing nanoparticles from plants and microbes is another crucial consideration. Freeze-thaw, heating, osmotic shock, and enzymatic lysis are physicochemical techniques that can be used for this purpose but are too expensive to be used on an industrial scale. These techniques can change the size, shape, aggregation, and structure of nanoparticles (Iravani,2011).

2.18.Toxicological Aspects of Silver Nanoparticles

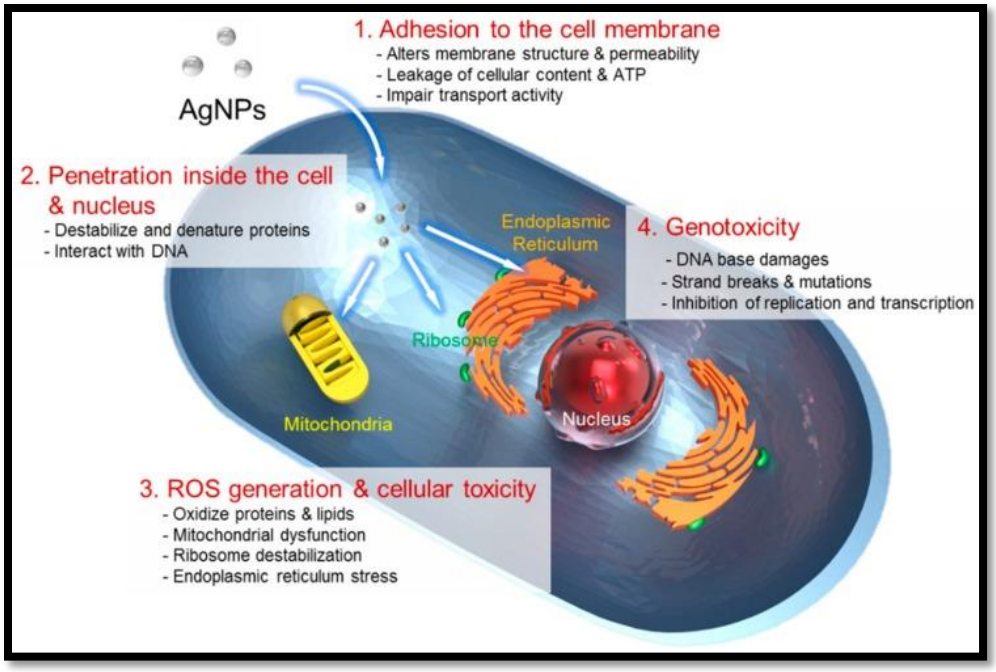
2.18.1. *In Vitro* Toxicology Studies on Ag-NPs

Worldwide, the use of nanotechnology in a wide range of commercial products has developed quickly. However, there is still a dearth of knowledge regarding the rise in the exposure of people, animals, and the environment to nanoparticles, particularly Ag-NPs, as well as the potential dangers associated with

their short and long-term toxicity. In vitro studies have revealed Ag-NPs' negative and toxic effects on cells or bacteria after exposure to these nanoparticles, in addition to proving the favorable capabilities of Ag-NPs, such as antibacterial and antifungal activity. Based on particular sizes, shapes, and densities, this is decided. By diffusing across membranes, this causes nanoparticles to gather and clump together at certain locations in target cells or organs, producing a colorimetric result. As a result, conventional results from nanoparticle *in vitro* experiments are less trustworthy since the cellular uptake data is misinterpreted (Antony *et al*., 2015).

Oxidative stress and genotoxic effects have been used to describe the cytotoxic effects of silver nanoparticles. Oxidative stress and genotoxic effects are brought on by the generation of reactive oxygen species (ROS), which is induced by the uptake of Ag-NPs by cells. Large-scale increases in ROS production cause cell death via apoptosis or necrosis (Carlson *et al*., 2008).

Generally speaking, in vitro investigations have indicated that host cells or bacteria that have been exposed to Ag-NPs suffer negative health effects. Reactive oxygen species (ROS) are principally responsible for Ag-NP-dependent cytotoxicity *in vitro* tests. Ag-NPs' cytotoxicity and genotoxicity are primarily determined by their size, concentration, and the length of exposure. In response to exposure to silver nanoparticles, lipid peroxidation, increased expression of ROS-responsive genes, and increased amounts of their proteins result, all of which contribute to DNA damage, apoptosis, and necrosis (Du *et al*.,2018).



Figure(2.6): The four main routes of cytotoxic mechanism of AgNPs. 1, AgNPs adhere to the surface of a cell, damaging its membrane and altering the transport activity; 2, AgNPs and Ag ions penetrate inside the cell and interact with numerous cellular organelles and biomolecules, which can affect corresponding cellular function; 3, AgNPs and Ag ions participate in the generation of reactive oxygen species (ROS) inside the cell leading to a cell damage and; 4, AgNPs and Ag ions induce the genotoxicity. (Lee and Jun, 2019).

2.18.2.Toxicity of Ag-NPs on Human Cell Lines

The resemblance of cell lines to parent tissues, their low cost, and their simplicity of use and culture have revolutionized scientific study. Additionally, the use of such cell lines in research eliminates the moral dilemmas related to the use of animal and human tissues because they offer an endless supply of biomaterials (Kaur and Dufour, 2012). Due to their high cost, high invasiveness, and decreased activity of several essential enzymes, immortalized hepatic cell lines are frequently utilized in investigations in place of liver biopsies. Additionally, there is no method that enables the preservation of liver biopsies in culture for time-consuming

experiments at the same time. The hepatic cell lines are described as having "epithelial morphology" in the database descriptions (Settivari *et al*., 2017).

The use of immortalized hepatic tumor cell lines has become a regular practice for the study of cancer. The most frequently utilized of these cell lines is Hepatocellular carcinoma, which was derived from a variety of cancers and has acquired popularity for its many uses in scientific research (Goyak *et al*., 2010). HepG2 cells were the first hepatic cell line to display the essential traits of hepatocytes. HepG2 often known as hepatoma, was the name given to this line when it was first isolated in 1975 (Tanimizu and Mitaka, 2016). The HepG2 cell line is used to examine the harmful effects of heavy metals, nanoparticles, and medications in vitro because it is thought that it has retained the majority of the metabolic processes of normal hepatocytes (Nikolic *et al*.,2018).

Ag NPs have strong anticancer properties as well. The use of biosynthesized silver nanoparticles for cancer therapy may be a successful substitute for the methods used to treat cancer today. These nanoparticles harm or shield healthy cells from malignant cells, damaging cancer cells, oncogenes, and healthy cells (Ahmad *et al*.,2019).

According to (Fehaid *et al*., 2020), silver nanoparticles (Ag-NPs) exhibit a wide range of biological actions, including anti-inflammatory, anti-bacterial, and antifungal properties. Apoptosis, necrosis, and DNA damage are all brought on by oxidative stress brought on by Ag-NP exposure. As a result, Ag-NPs have been demonstrated to cause cytotoxicity in a number of cancer cell lines, such as prostate cancer cells (Firdhouse and Lalitha, 2013), human Chang liver cells, rat basophil leukaemia (RBL) cells, lung carcinoma A549 cells (Gurunathan *et al*., 2015), MCF-7, and HCT-116 cells (Khan *et al*., 2021). Additionally, it has been

demonstrated that Ag-NPs injure cancer cells more than non-cancerous cells (Faedmaleki *et al*., 2014).

2.19.Mechanisms of Antibacterial Action

Currently, the research mostly supports three methods (Marambio-Jones and Hoek, 2010; Daka *et al*., 2016; Qing *et al*., 2018) through which Ag-NPs exert their antibacterial function. According to the first theory, Ag-NPs affect cells at the membrane level because they can pass through the outer membrane and accumulate in the inner membrane, where their adhesion causes the cell to become damaged and destabilized, increasing membrane permeability and leading to cell death (Ivask *et al*., 2014; Seong and Lee, 2017).

Additionally, there is evidence that Ag-NPs can interact with sulfur-containing proteins in bacterial cell walls, which may result in structural damage and cell wall rupture. The second mechanism suggests that nanoparticles can enter cells, where it has been suggested that Ag-NPs will have an affinity to interact with sulfur or phosphorus groups present in intracellular content such as DNA and proteins, changing their structure and functions. Nanoparticles can also break and cross the cell membrane, altering its structure and permeability. Similar to how they can damage intracellular machinery, activate the apoptotic pathway, and change the respiratory chain in the inner membrane by reacting with thiol groups in the enzymes and creating reactive oxygen species and free radicals. The release of silver ions from the nanoparticles, which is thought to happen concurrently with the other two mechanisms, they can interact with biological components, changing metabolic pathways, membranes, and even genetic material because of their size and charge. (Agnihotri *et al*.,2013; Ivask *et al*.,2014).

2.20.Unfavorable Effects of Ag-NPs

Due to their use in a variety of commercially available items, silver nanoparticles (Ag-NPs) are one of the nanomaterials that the majority of us have already come into touch with (Dlugosz *et al*., 2021). It is necessary to accurately estimate the negative effects of Ag-NPs and comprehend the health risks associated with the interaction of silver nanoparticles with the human body and ecosystem because they are already widely used around the world but we are not fully aware of their toxic effects or their safety (Li and Cummins, 2020). The potential for Ag-NPs to be discharged into surface waters and, as a result, have an influence on aquatic creatures is related to the growing use of silver nanoparticles as antiseptics (Furtado *et al*.,2016; Gagnon *et al*.,2021). Ag-NPs may influence a health risk is created by the ecosystem's nitrogen cycle and the possibility that it enters the food chain (Jiang *et al*., 2017).

The toxicity of nanoparticles varies with their concentration, size (Kong *et al*., 2020), surface charge (Ivask *et al*., 2014), shape (Beer *et al*., 2012), method of synthesis (Vasanth, and Kurian, 2017), functionalization of their surface (De Lima *et al*., 2012), time and route of administration, as well as the tested model or individuality of each organism (De Lima *et al*., 2012). The negative effects of silver nanoparticles can manifest as moderate skin and eye discomfort. According to (Swidwinska-Gajewska and colleagues, 2014), Moreover, Ag-NPs may trigger skin allergies. Ag-NPs are used as a wound dressing (Lee *et al*.,2010; Samberg *et al*., 2010).

2.21. Applications of Green Synthesized Silver Nanoparticles

Due primarily to their antibacterial and antifungal capabilities, silver nanoparticles have a wide range of uses that have revolutionized applied medicine.

AgNPs have been widely used in therapeutic applications such cardiovascular implants, catheters, dental composites, and nanobiosensing as wound dressings and creams or as an antibacterial covering (Cortivo *et al*., 2010). Burns and chronic ulcers are just a couple of the ailments that are treated clinically with silver nanoparticle wound dressings (Chaloupka *et al.,* 2010). These wound dressings dramatically cut the healing period of the damage when compared to the gauze dressing or cream that was previously used and contained 1% Ag (Huang *et al*.,2007; Johnson *et al*., 2020).

They also increased the bacterial clearance of contaminated injuries. The use of chitin-AgNPs in wound dressings had the potential to treat wounds by reducing bacterial growth (Singh and Singh, 2014). It was chosen to employ AgNP in place of the Ag element since the silver-coated silicone heart valve caused an allergic reaction and prevented the patient's fibroblasts from operating properly (Grunkemeier *et al*., 2006). The surface coating of cardiac valves and stents with silver nanoparticles proven to be a safer, non-toxic, and antimicrobial option. Furthermore, the addition of nanoparticles to the heart valves' polymer core boosts their biocompatibility and calcification resistance (Ghanbari *et al*., 2009).

The development of Nano biosensors, which are utilized for illness diagnosis, therapy monitoring, cell tracking, and in vivo detection of Nano probes, was made possible by the physicochemical properties of silver at the Nano scale (Marchiol, 2012). Catheters are extremely vulnerable to contamination in a typical hospital setting. AgNP coating was non-toxic and prevented the growth of biofilms in catheters, successfully lowering the amount of bacteria to 72 h (Roe *et al*., 2008).

To make sterile medical apparel that prevents or reduces contamination with dangerous bacteria like *S. aureus*, the textile industry uses silver non-toxic nanoparticles with antimicrobial properties. Dental applications for AgNPs have also been discovered. AgNPs, for instance, as a component of orthodontic glue, increase bacterial resistance, enhancing the adhesive's bond strength (Marchiol, 2012; Akhavan *et al*., 2013), and as a coating for dental equipment, lowering microbial colonization and enhancing antifungal activity (Roe *et al*., 2008, Magalhães *et al*., 2012).

Plant extract-produced AgNPs exhibit an anti-diabetic potential. In alloxan-induced diabetic rats, blood glucose levels were lowered by silver nanoparticles made from Solanum nigrum leaf extract. AgNPs demonstrated a hypoglycemic impact in comparison to glibenclamide, the typical anti-diabetic medication (Sengottaiyan *et al*., 2016).

Additionally, Argyreia nervosa leaf extract was used to create silver nanoparticles that shown antidiabetic action. They prevented two digestive enzymes, -amylase and -glycosidase, from working (Saratale *et al*., 2017). Consumer goods like water filters, deodorants, soaps, socks, and room sprays all contain AgNPs (Manjumeena *et al.*, 2014).

In the past few years, there has generally been a lot of work done to improve green synthesis. Because green synthesis is more affordable, environmentally friendly, nontoxic, and capable of being scaled up for large-scale synthesis than chemical and physical processes, it is superior to them. The use of metal nanoparticles in nanotechnology and, concurrently, a wide range of practical applications have been made possible by the growing understanding of the use of

green synthesis to manufacture metal nanoparticles, particularly AgNPs (Ahmed *et al*., 2022)

Chapter Three
Materials and Methods

3. Materials and Methods

3.1. Materials

Equipment, chemicals and tools used in the present study with their manufacture are listed in tables (3.1, 3.2, 3.3, 3.4 and 3.5)

Table (3.1): Devices and equipment used in the present study

Device and Equipment Name	Manufacture Company
Freezer -20	Bush/UK
96-Well Microtiter™ Microplates	Thermo Fisher Scientific U-bottom tray/Japan
Autoclave	Farazmehr/ Isfahan, Iran
Beakers Glass Wear (200ml)	Mehravar Kish.Co./Iran
Centrifuge	Behdad, Tehran/Iran
Conical Centrifuge tube(falcon tube)	Fisher Scientific/UK
Cuvette Washer	Sigma–Aldrich/ UK
Digital balance	ES-1000H/China
Digital scale	Standard Instruments CO./ Hong Kong
Distiller	GFL/Germeny
DSR1 Desk Sputter Coater	Nanostructured Coatings Co./ in Iran
Dynamic light scattering (DLS)	Malvern Instruments Ltd./ UR
ELISA reader device	BioTek/ USA)
Eppendorf Pipette(1000,Blue, PP)	Trustmomed/ Germany
Eppendorf® Concentrator Plus	Eppendorf®/ (EU)
Funnel	Mehravar Kish.Co.Iran
HEPG2 cells	(NCBI C158)
Hydro distillation (Clevenger)	Open Sanctions/Iran
Incubator	Behdad, Tehran/Iran
Laboratory Film	Bemis/Fisher Scientific

Low-volume quartz cuvette ZEN2112 for DLS test	Hellma Analytics/Malvern, UK
Malvern Instruments for DLS test	Malvern/ UK
Micropipette (100, 1000 µl)	Sciences/Flinn Scientific
Plastic Pitridish	Huida Medical/Iran
Plastic Tubes (50ml)	Huida Medical/Iran
Refrigerator	Behdad, Tehran/Iran
Shaker incubator	Pars Azma, Tehran/ Iran
UV-visible spectrophotometer	Shimadzu/ Japan
Sterilized Cotton Swabs	Medmatrix Global
Syringes 1, 5, 10, 20 ml	Kompass database
TESCAN MIRA3 for FESEM	Czech Republic
Thermometer	Hartwig instruments/
Tip in different size	Trustmomed eppendorf pipette/Germany
Water Bath	ProfiLab/Germany
Millipore filter 0.45mm, 0.22mm	Sigma–Aldrich/ UK
Micro and cooling centrifuge	Hermle Labortechnik/ Germany
Light microscope (Olympus CK 40)	Tokyo/ Japan

Table (3.2): List of Chemicals, Reagents and their Suppliers

Chemicals	Supplier
Silver nitrate ($AgNO_3$) Reagent Grade	Carolina Biological
0.9% NaCl saline	Golden Horse Medical Supplies/Philippine
70% ethanol alcohol for Drying cuvette in DLS	
Phosphate buffered saline	SAMA Tashkhis/Iran
Arginine(0.001 M)	Sigma Aldrich /UK
sodium chloride(0.15 M)	
Antibiotics discs $Amikacin_{30}\mu g(AN_{30})$, $Ceftriaxon_{30}\mu g(CRO_{30})$, and $Ampicillin_{10}\mu g(AM_{10})$	PADTAN TEB/Iran
5% Wash cuvette solution of Hellmanex	Hellma Analytics/USA
Ultra-pure water for wash cuvette	Pasteur Institute of Iran.
Serum-media(Used for growth cell and inactivate Trypsin EDTA)	Sigma Aldrich/ USA
Diethyl either	
Phosphate buffer saline (PBS)	Bioworld/ USA
Glycerol	Sigma/ USA
Phosphate buffer saline(PBS)	Bioworld /USA
H_2SO_4	Puritan/USA
$BaCl_2$	Hebei Yanxi Chemical Co., Ltd./China

Table (3.3): List of Culture Media, Preparation, Purpose, Type and Company

Name of Media	Purpose of Media	Type of Media	Manufacturer Company
Brain Heart Infusion Broth	For activation and maintenance of ATCC bacterial isolates. (MacFaddin,2000)	Enriched Medium	Himedia /India
Blood Agar	For Activation Streptococcus bacteria (MacFaddin,2000)	Enriched Medium	
Muller Hinton Agar	used for Antimicrobial Susceptibility Testing (AST) for bacterial isolates. (MacFaddin,2000)	Susceptibility Test Medium	Sigma Aldrich/USA
Muller Hinton Broth	It is recommended to use it for broth dilution MIC studies. (MacFaddin,2000)	Susceptibility Test Medium	
Serum-medium	Used for growth cell and inactivate Trypsin EDTA (Hodges *et al*.,1977)	To detach adherent cells	Gibco/ U.K

Table (3.4): Antibiotic discs

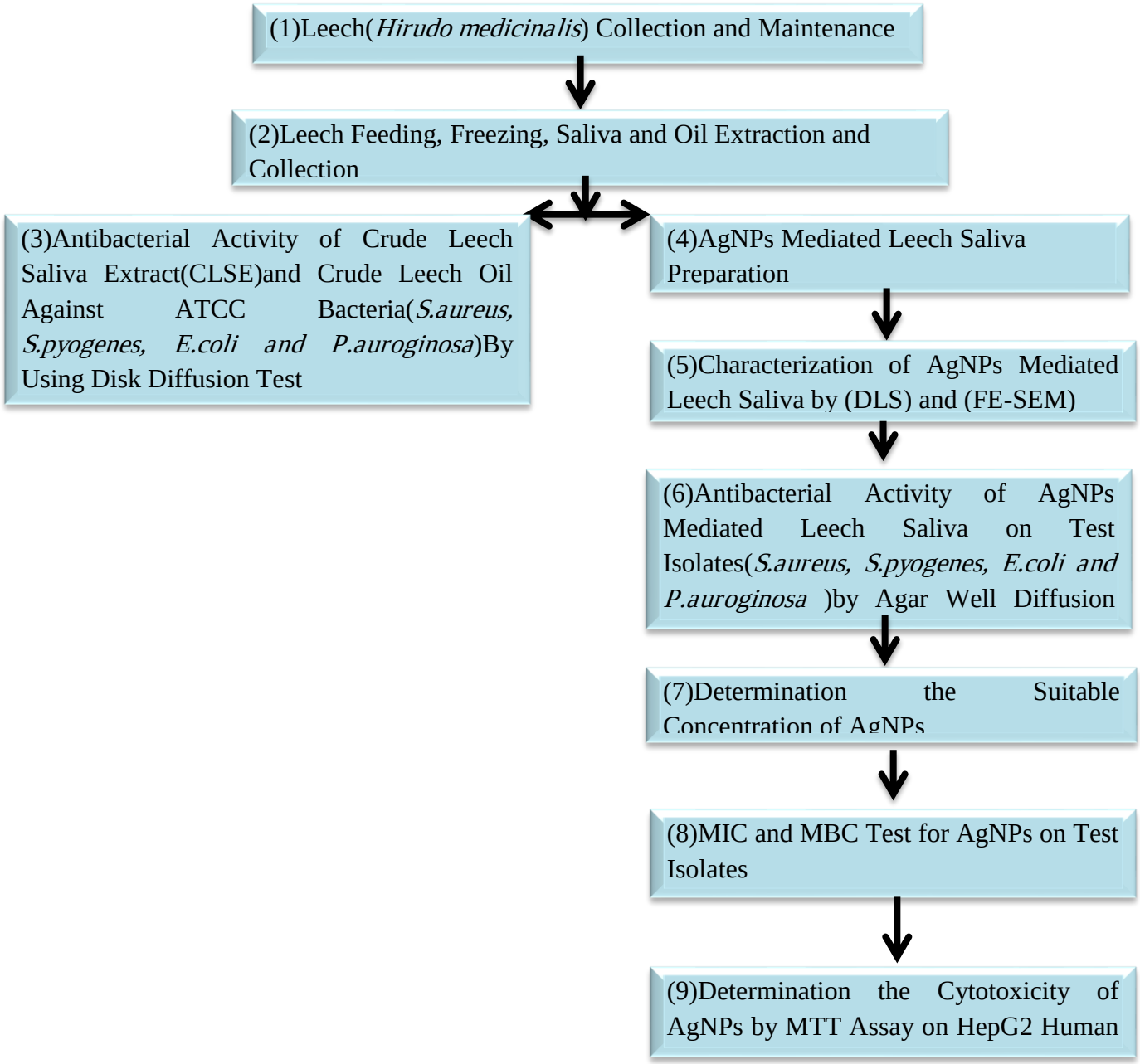
Antibiotic class	Antimicrobial agent	Symbol	Disk content (µg) (potency)	Mode of action
Penicillins	Ampicillin	AM	10	Cell wall synthesis inhibitors
Aminoglycosides	Amikacin	AN	30	Protein synthesis inhibitor
Cephalsporin	Cefetriaxone	CRO	30	Cell wall synthesis inhibitors

Table (3.5): MTT Kit

MTT kit	Company
MTT solution 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl -2H- tetrazolium bromide	Thermo Fisher Scientific/USA
Solubilization solution: 40%v/v Dimethyl formamide 2% Glacial Acetic Acid 16% Sodium Dodecyl Sulfate	Sigma Aldrich/USA

3.2. Methods

3.2.1. Experimental Design



Figure(3.1) Experimental Design

3.2.2. Area of Study

The period of present study is extended from February 2022 to May 2023 and this study is performed in Malaria and Vector Research Group (MVRG)/Biotechnology Research Center (BRC)/Pasteur Institute of Iran. Leeches were grow up using rice from the Bandar-e-Anzali rice field in Iran's north. The climate in that location is similar to a subtropical region, with average annual temperatures of 16.1^0Cand adequate rainfall throughout the year of about 1496 mm.

Hirudo medicinalis species was chosen for testing because natural saliva plays a significant role in clinical criteria. Based on its physical differences from other species, this species can be identified. *Hirudo medicinalis*, according to Solgi *et al*., (2021) has comparatively elongated spots, a cylindrical, ventrally flattened body with a short anterior sucker, and dark brown on the dorsal side**.**

3.2.3. Leech Sampling

The Bandar-e- Anzali rice farm in northern Iran's city provided the leeches, which were subsequently processed and housed in well-aerated plastic containers filled with non-chlorinated tap water in a separate room at room temperature. Every two days, the water was changed routinely (Abdulkader *et al*., 2013).

3.2.4. Maintenance of Medicinal Leech

Typically, special boxes containing moist peat are used to distribute the farmed therapeutic leeches. After being delivered to the relevant staff, they should be carefully cleaned, and any associated dead specimens and soil particles should be removed. Leeches are then moved to glass, clay or plastic containers that are half-filled with de-chlorinated water, with calcium content and kept at a

temperature of 12 to 25 °C (Rassadina *et al*., 2006), Figure(3.2A). Usually, a three-liter container may hold up to 50 leech. According to Abdulkader *et al*., (2013) overcrowding causes important functions to be suppressed and raises mortality. A piece of thick linen cloth or a tight-fitting plastic lid with tiny holes is used to cover the container snugly, providing the essential ventilation. Once or twice a week, the container's water should be changed, and once a month, it should be properly cleaned without the use of chemicals. The container with leeches should be placed in a dark and cool place , free of sharp odors and vibration, leeches can survive for up to a year and a half (Abdulkader *et al*., 2013).

3.2.5. Preservation and Maintenance of Standard Bacterial Isolates (ATCC) bacteria

The ATCC bacterial strains from the Institute Pasteur of Iran's bacterial bank (*S. pyogenes* ATCC104030, *S. aureus* ATCC25923, *P.aeruginosa* ATCC27853 and *E.coli* ATCC25922) that were utilized in this work. Immediately after receiving dried cultures, revive them by thawing them out and then adding a suitable growth medium (BHI) broth to them. After an overnight incubation period, add 2 ml of sterile glycerol to the bacterial eppendorf tubes and store them between -70°C and -80°C. At 4°C or lower, freeze-dried cultures can be stored without risk. Rehydrate or dilute cultures after receiving them, using the recommended medium and incubation conditions listed for the product. Live cultures should be placed in an adequate growth medium as soon as they are received and incubated according to the product's instructions (Reddy, 2007).

3.2.6. Preparation of Solutions

3.2.6.1. Phagostimulatory Solution

Dissolve 0.001 M of arginine and 0.15M of sodium chloride in 10ml distaled water, warm the solution to 37^{o}C when use it for feeding the leech, this temperature help leech for feeding attraction (Abdualkader *et al*., 2013).

3.2.6.2. MacFarland Solution

A 0.5 McFarland standard may be prepared as describe below:

Add a 0.5ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.18 mol/liter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard. Transfer the barium sulfate suspension in 4 to 6ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums. Tightly sealed the tubes and stored in the dark at room temperature. This slandered solution was used to visually compare the turbidity of bacterial suspension (CLSI, 2021).

3.2.6.3. Trypsin-(EDTA) solution

It was prepared by dissolving 15gm of trypsin-EDTA in 125 ml of D.W and constantly added by stirring the volume completed to 1 liter, and then filtration by using 0.22µm millipore filters and stored at (- 80˚C). These solution was used to detach and disaggregate the adherent monolayer cells from the bottom of the culture vessel in cytotoxicity assay (Hodges *et al*.,1977).

3.2.7. Media preparation and sterilization technique

All culture media presented in table (3.4) were prepared according to the manufacturing company instructions .The constituents were dissolved in distilled water completely , sterilized by autoclaving at 121^{o}C for 15min at 15 pound/inch^{2}. After cooling to 45-50^{o}C each medium was dispensed into sterile petri dishes in case of agar media and in sterilized screw tubes in the case of broth media . The work performed in laminar flow cabinets . Then , poured media were incubated for 24hr at 37^{o}C to ensure sterility. (McFaddin , 2000)

3.2.8. Leech Saliva Extraction

For the duration of the experiment, leeches were housed in plastic containers filled with water at 23°C. Three days later, the water was changed. Twelve weeks were spent starving leeches. A method published in the literature by Abdualkader *et al*., (2011) and Ojo *et al*., (2018) was slightly modified in order to acquire saliva. Eighty leeches(80) were used in this study. In a nutshell, parafilm membrane was stretched over a falcon tube containing 10 ml of distill water, 0.001 M arginine (0.02g), and 0.15 M (0.08g) saline solution. The solution was then put in the newly invented device Figure(3.2B). Leeches were allowed to suck until satiation. Directly after leeches drop down from the membrane, they were immobilized by putting them in plastic container surrounded by ice for 10-15 min as show in figure(3.2C). This technique forces the leeches to vomit whatever they have sucked. To complete the saliva collection, leeches were squeezed smoothly from the posterior toward anterior (mouth) sucker Figure(3.2D). All fluids that have been vomited were collected (bloody fluids were discarded) in clean eppendorf pipette test tubes. Collected fluid was centrifuged at 4^{o}C, 9000 rpm for 10 minutes.

Supernatant obtained after centrifugation was referred to as crude leech saliva extract is preserved at −4°C in a refrigerator.

Figure(3.2): (A) Leech (*Hirudo medicinalis*) are kept in container.(B) Leech while sucking the solution through the membrane. (C) Leech freezing on ice bag.(D) Leech were squeezed smoothly from the posterior toward the interior sucker to complete saliva extraction.

3.2.9.Leech Oil Extraction

In order to extract the oil, twenty(20) leeches were prepared. The head and posterior sucker of the leech were cut by a surgical scalpel. The abdominal contents were emptied and washed 3 time using water. After drying in the vicinity of air, it was turned into smaller pieces using in the mortar and pestle until a homogenous suspension was obtained, then centrifuged for 20 min at 3000 g , the liquid was filtered by using Millipore filter 0.45 mm and the crude extract was stored at 4°C until the oil extraction was studied. Oil was obtained by using the

distillation system in the Clevenger machine. In this way 10 g of leeches' pieces, 100 ml distilled water and diethyl ether were used for each extraction run. Then the oil was obtained by using vacuum rotary evaporated apparatus, until all the diethyl ether was completely evaporated, leaving the absolute essential oil and stored in a glass flask at 22^{o}C Figure (3.3) (AL-Jumilly *et al*., 2017). In the next step susceptibility test based on the disc diffusion was performed according to the standard protocol.

Figure(3.3):Leech Oil Extraction Using Clevenger Machine based on the Distillation System.

3.2.10. Antibacterial Activity of Crude Leech Salivary Extract and Leech Oil Extract

The Antibiotic susceptibility testing is an *in vitro* test that uses the diffusion technique on agar media to determine how sensitive a bacterium is to one or more antibiotics. The data will be used to track bacterial resistance to antibiotics and to assist the clinician in selecting the best medication to treat a bacterial infection.

Antibacterial activity of CLS , crude leech oil extraction and Ag NPs mediated CLS were assessed by using a disc diffusion test, MIC and MBC test methods as described by (Coyle *et al*., 2017).

Inoculum preparation (adds bacterial colonies to the Phosphate buffer solution, compare the resulting suspension to the McFarland standard(0.5). This is done by holding both the standard and the inoculum tube side by side and no more than 1 inch from the face of the Wickerham card (with adequate light present) and comparing the appearance of the lines through both suspensions.

Paper filter discs (saturated tablets with CLS and leech oil extract overnight and stored in refrigerator).

3.2.10.1. Inoculum Preparation

1. At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 5 ml of a Mueller Hinton broth medium.

2. The broth culture was incubated at 37°C overnight.

3. Five ml PBS was autoclaved for each 4 bacterial isolates of the present study. The inoculum was done to prepared turbidity similar to 0.5 McFarland by using from an overnight culture (CLSI, 2021).

3.2.10.2. Screening for Antibacterial Activity of Crude Leech Salivary Extract and Crude Leech Oil Extract

Both gram positive and gram negative microorganisms were included in this assay.

1- *S. aureus* ATCC(25923), *S. pyogense* ATCC(104030), *E. coli* ATCC(25922) and *P. auroginosa* ATCC(27853). These bacteria's inoculum was collected from the Tehran/Iran Institute Pasteur's bacterial bank.

2- Amikacin 30µg and Ampicillin10µg were utilized as appositive control. A tube containing 5 ml of phosphate buffer solution was used to prepare the inoculum. The suspension's turbidity was set to the McFarland level of 0.5 (1.5×10^8 CFU/ml). The McFarland standard and inoculum tubes were adjusted by positioning them in front of a white sheet of paper with black markings. Inoculum was applied right away and 18–24 hours later It was prepared afterwards.

3- A Mueller-Hinton agar (MHA) plate received a 50 µl bacterial inoculum. In order to allow the agar medium to absorb the excess water favor, the plate was maintained at ambient temperature. In order to allow the agar medium to absorb the excess water favor, the plate was maintained at ambient temperature. This was accomplished by putting the dish in an incubator and leaving the lid slightly half closed. The ideal MHA thickness is 4 mm.

4- Filter paper disc saturated with CLS solution and crude leech oil extract after the medium has solidified. The plate was then incubated for 18 to 24 hours at 37 °C. Using a caliper, the zone of inhibition that formed around each well was measured.

5- The halo of growth inhibition that is produced around the antibiotic disc is used to gauge a bacterium's sensitivity. Each antibiotic has a unique range of inhibition for bacterial growth. Measure the width of the absence of development using a ruler to estimate the sensitivity or resistance, and then use the table (CLSI, 2021).

3.2.11. Synthesis of Leech Salivary Extract-Mediated Silver Nanoparticles

Synthesis is done via a chemical reduction process**.** A solution of silver nitrate (0.1mM) was prepared by dissolving 0.015 g of the salt in 1 ml of sterile distilled water. Necessary calculations were done to determine the appropriate values according to the $AgNO_3$ molecular weight.

So silver nitrite were prepared in different final concentrations as follows: (0.004, 0.006, 0.1, 0.6 mM), (Pratama *et al*., 2019)

1- (1 ml) of crude leech saliva was combined with 0.1ml for each concentration of prepared $AgNo_3$. pH of supernatant was adjusted to (8) and then incubated for 48 hrs. at 37^{o}C in a shaking incubator (150 rpm) in aerobic condition (Singh *et al.,* 2014).
2- After that the reaction mixture was centrifuged at (10000 rpm , 4 C° for 10 min) , the supernatant was discarded and the sediment was taken . In order to purify saliva nanoparticles. These sediment was washed with DDW , these

step were repeated three times. The final suspension was dried in oven at 40 C° for 18-24 hrs.

3- The production of nanoparticles was accompanied by a 48 hour change in color from colorless to brown. Only two from four concentrations of $AgNO_3$ (0.1, 0.6mM) show the change to brown color figure(4.3A, B).
4- The Ag-NPs were then moved to a micro tube that had been light-protected (i.e., wrapped in aluminum foil) and was kept at $4C^0$ for further analysis (Dhanjal and Cameotra , 2010) .

3.2.12. Characterization of Biosynthesized LSE-AgNPs

The physical characteristics of LSE nanoparticles were characterized by **DLS** and **FE-SEM** for two concentrations of $AgNO_3$ (0.1, 0.6mM). Accepted results were obtained from (0.1mM) compared with the second concentrations(0.6mM) that show large size of nanoparticles and particles between(90-890nm), So we select the first one for further analysis.

3.2.12.1. Dynamic Light Scattering

Dynamic Light Scattering, sometimes referred to as photon correlation spectroscopy (PCS), is a particularly effective method for examining how macromolecules diffuse in solution. The hydrodynamic radii that can be determined from the diffusion coefficient depend on the size and shape of the macromolecules. The homogeneity of proteins, nucleic acids, and complexes of protein-protein or protein-nucleic acid preparations can all be studied using DLS, as can interactions between proteins and tiny molecules. An appropriate detector picks up the signal after the sample is subjected to a monochromatic beam of light in a typical light-scattering experiment. Light scatters in all directions when a

monochromatic beam of light interacts with a solution containing macromolecules, and this scattering depends on the size and form of the macromolecules. Analysis of the scattered light's intensity as time-averaged intensity in static light scattering yields useful data on the molecular weight and gyration radius of macromolecules (Stetefeld *et al*., 2016).

The samples which used for DLS test must be prepared in solution. If it is desired to determine the electric charge of the particles, it is better to prepare samples in water containing a small amount of salt. A general salt like NaCl can be used but usually chloride ion is absorbed by nanoparticle and causes disorder. It is recommended to use nitrate salt (KNO_3) so that 10 mM KNO_3 is ideal for all concentrations of particles. The solution which is prepared for the DLS test should be clear to very slightly hazy. If the prepared solution was white or too hazy ,it should be diluted as suitable (https://www.research.colostate.edu/wp-content/uploads/2018/11/Guide_for_DLS_sample_preparation.pdf).

Saliva from Ag- NO_3-leech, Malvern Instruments Ltd., in England, performed a test to determine the nanoparticle size using dynamic light scattering (DLS), and report two deals with determining the zeta potential Appendix (1,2) (Babayi *et al*., 2022). As follows are the analysis conditions:

Dispersant: Water **Temperature**: 25^{o}C **Viscosity**: 0.887 **Measurement position** (mm): 5.50

3.2.12.2. Field Emission Scanning Electron Microscopy (FE-SEM)

The high- technique known as field emission scanning electron microscopy (FE-SEM) is used to examine the topography of nanomaterials, including the size and form of nanoparticles. It is important that the surface of the samples is conductive for imaging with an electron microscope. The samples should have a layer of conductive metals produced for this reason.

1- In the initial step, we dropped a drop of nanoparticle onto the slide to dry before moving this sample to the Desk Sputter Coater (DSR1) equipment in order to characterize the created nanoparticles in this study.
2- DSR1 is produced in Iran by Nano-Structured Coating Co. Using the Desk Sputter Coater (DSR1), a thin layer of gold (Au) was applied to the sample in this step.
3- Then, TESCAN MIRA3 (15.0 KV) was utilized to take the image picture of the samples' surface. Images were captured at 10 KX (10.000 x) and 35 KX magnifications. 95% confidence was used when conducting the test (Jaganathan *et al*., 2016).

3.2.13.Screening Antibacterial Activity of Silver Nanoparticles Mediated by Crude Leech Salivary Extract

The antibacterial activity of crude leech salivary extract mediated silver nanoparticles on test isolates was investigated using the agar well diffusion method according to (CLSI, 2021). The LSE-Ag at 15 µl was tested using this method. A 4mm thick layer of Muller Hinton agar (MHA) was created.

Nanoparticles were prepared in different concentrations as follows: **1-**100 µg/ml, **2-** 200 µg/ml, **3-** 300 µg/ml, **4-** 400µg/ml.

15 microliters of the produced nanoparticles were employed in the most recent antibiogram, which included analysis.

1- Turbidity of each bacterial isolates compared to McFarland 0.5 standard to get the right concentration for each of them .
2- By using sterile cotton swab bacterial inoculum of *S. aureus, S.pyogenes*, *P.aeroginosa* and *E. coli* were seeded on the agar plate.
3- A sterile cork borer (5 mm in diameter) was used to drill four holes on the plate.
4- The four holes each filed 15 µl of a CLSE-AgNPs (four concentrations).
5- Ceftriaxone 30µg against *E. coli* and *P.aeroginosa*, Ampicill in 10µg against *Staphylococcus aureus* against *S.pyogense* as positive control were utilized.

6- After that, the plates were incubated for 24 hours at 37 °C, during which time zones of inhibition were seen, quantified, and recorded (CLSI, 2021).

3.2.14.Determination of Minimum Inhibitory Concentration of Leech Salivary Extract Nanoparticles (LSE-AgNps) by Agar Well Diffusion Test

In the last well diffusion, 15 microliters of the prepared Nanoparticles were used and analyzed. According to the last antibiogram results, we found that the suitable concentration of nanoparticle is 100µg/ml. Therefore, in order to determine the minimum inhibitory concentration (MIC) of bacteria, serial dilutions of solution were prepared (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.13µg/ml) respectively.

1- 10 µl of each solution was added to Mueller Hinton's culture medium inoculated with bacteria such as *S. aureus*, *S.pyogense*, *P.auroginosa* and *E. coli.*
2- Turbidity of each bacterial isolates compared to McFarland 0.5 standard to get the right concentration for each of them.
3- The method of determining the sensitivity or resistance of a bacterium is based on the halo of growth inhibition that is formed around the antibiotic disc.
4- According to each antibiotic, each bacteria has specific inhibition growth distance. To determine the sensitivity or resistance, it is enough to measure the diameter of lack of growth with a ruler and then determine by referring to the table (CLSI, 2021).

3.2.15. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Leech Salivary Extract-Mediated Silver Nanoparticles By Microtiter Dilution Method

1- Simply put, Mueller Hinton broth was newly produced, sterilized, and injected with the necessary bacteria.
2- It was then incubated in 37°C for 18 hours. We prepared McFarland 0.5 turbidity standards from each microbe the following day. The turbidity of bacterial suspension was compared and matched with the turbidity of 0.5 McFarland units.
3- According to the numbers of the desired bacteria 4 rows of 96-Well Microtiter™ Microplates were assigned for MBC test. Each row was used for one bacterium. Column one was negative control which contain of 100µl Mueller Hinton broth only. Column two was positive control (growth

control) which contains of 100µl Mueller Hinton (M.H) broth inoculated with suspension of (0.5) 1.5 $\times 10^8$ CFU/ml of each bacterium. Column three was containing of 100µl Mueller Hinton broth which have mixed with 100µl prepared LSE-AgNPs. Columns four to seven were contained 100µl Mueller Hinton broth.

4- In order to preparation of serial dilution 100µl of the well three was removed and mixed with the next well. In this way, dilutions were prepared until 1/16.
5- Finally were removed out 100µl of diluted M.H from last column.
6- A 10 microliter of bacterial inoculum were added to each well , except all wells of (column 1).
7- In order to determination of MIC, Microtiter plate incubated at 35 ± 2°C for 24 h and subsequently turbidity caused by bacterial growth examined visually. OD at 570 nm was recorded spectrophotometrically . MIC was determined as the lowest LSE-AgNPs concentration showing absence of growth as compared with the growth in the LSE-AgNPs -free well.
8- Finally in order to determination of MBC all content of each not grown well's moved to M.H agar plate and incubated at 35 ± 2°C at the following times 24 h for *P.aeruginosa* and *E. coli* which were rapidly growing Gram-negative rods and48 h for *S.aureus* and *S.pyogenes*. When there was no growth, it was considered bactericidal, and when there was growth, it was considered bacteriostatic (Omeje and Kelechi, 2019).

3.2.16. Cell Viability with MTT Assay Protocol

3.2.16.1. Principle

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) calorimetric assay measures the capacity of live cells to transform a soluble tetrazolium salt into an insoluble formazan precipitate. Tetrazolium salts, like NADH and NADPH, can absorb electrons from oxidized substrates or the proper enzymes. Succinate dehydrogenase activity specifically causes MTT to be decreased at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system. The yellow salts are changed into blue formazan crystals in this reaction, which can be dissolved in an organic solvent and whose concentration can be measured spectrophotometrically. MTT Assays (Supino, 1995) is cited.

An ELISA reader device may read the optical density (OD) produced by dissolving formazan crystals in an organic solvent such isopropanol. The concentration of formazan, which is inversely proportional to the metabolic activity of live cells, and optical density are directly related.

This assay is frequently used to detect the *in vitro* cytotoxic effects of medicines on cell lines or primary patient cells since for the majority of cell types the total mitochondrial activity is proportional to the number of viable cells (van Meerloo *et al*., 2011).

Hepatocellular carcinoma cell line (NCBI C158) from the cell bank of the Pasteur Institute of Iran were used in this investigation. The cells were defrosted, moved to a flask containing 10% FBS-DMEM growth media, and the flask was

then placed in an incubator at 37°C, 90% humidity, and 5% carbon dioxide concentration. Every three to four days, the culture media was replaced.

3.2.16.2. HepG2 is a human liver cancer cell line

The average diameter of the HepG2 cell is about 10–20 μm. Cells have large nuclei and contain 3–7 nucleoli. HepG2 accommodates low mitochondrial content and poorly developed smooth endoplasmic reticulum. Hepatocellular carcinoma are epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice. The cells secrete a variety of major plasma proteins, e.g., albumin, and the acute-phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen. They have been grown successfully in large-scale cultivation systems. HepG2 cells have adherent properties and grow as monolayers in small aggregates. (Moscato *et al*., 2015).

3.2.16.3. Hepatocyte

The typical hepatocyte is cubical with sides of 20-30 μm, The typical volume of a hepatocyte is 3.4 x 10−9 cm3. Smooth endoplasmic reticulum is abundant in hepatocytes, in contrast to most other cell types (Trefts, 2017). Hepatocytes display an eosinophilic cytoplasm, reflecting numerous mitochondria, and basophilic stippling due to large amounts of smooth endoplasmic reticulum and free ribosomes, brown lipofuscin granules are also observed these correspond to cytoplasmic glycogen and lipid stores removed during histological preparation, Hepatocyte nuclei are round with dispersed chromatin and prominent nucleoli. Anisokaryosis (or variation in the size of the nuclei) is common and often reflects tetraploidy and other degrees of polyploidy, a normal feature of 30-40% of hepatocytes in the adult human liver (Celton-Morizur *et al*., 2010).

3.2.16.4.Cell Line Maintenance

When the cells in the vessel formed confluent monolayer, the following protocol was performed (Geraghty *et al*., 2014) :

1- The growth medium was aspirated and the cell sheet washed with PBS.

2- Two to three ml trypsin/EDTA solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle, rocking. The vessel allowed incubation at 37 C° for (1 – 2) min until the cells were detached from the vessel.

3- Fresh complete (10% Fetal Bovine Serum-DMEM) growth medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.

4- Cells were redistributed at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37 C° in 5% CO_2 Incubator.

Cell concentration was achieved by counting the cells using the haemocytometer (Appendix 4) and applying the formula:

$$Total\ cell\ count/\ ml = cell\ count \times dilution\ factor(sample\ volume) \times 10^4$$

3.2.16.5. Sample Preparation

First, different concentrations of the sample, including 25, 50, and 100 µg/ml, were made using full culture media (10% FBS+DMEM), in order to study the toxicity of the samples and their impact on the growth and proliferation of cells figure(3.4)

Figure(3.4): Complete culture medium (DMEM + 10% FBS) containing of suitable concentration of leech saliva $AgNo_3$ nanoparticle.

3.2.16.6. Quantitative Toxicity Test (MTT)

In this work:

1- (rate of 1×10^4/well) HEP G2 cells, Appendix (4) and 100µl of culture media were first added to each well of a 96-well cell culture plate.
2- The cells were then incubated at 37°C for 24 hours to allow the cells to stick to the plate's bottom.
3- The culture medium covering the cells was removed as much as possible after ensuring that they adhered.
4- Then, 100µl of the produced concentrations were added to each culture well. Triplicates were used per each concentration as well as the control and the cells were allowed to remain close to these concentrations for an additional 24, 48, and 72 hours.
5- Following the removal of the culture media, 100µl of MTT 0.5mg/ml in PBS (M2128 Sigma Aldrich) was added to each well before being incubated for 4 hours. The purple crystals were dissolved with isopropanol after the solution

had been in the cells for 4 hours. The plate was shaken for 15 minutes to let the MTT sediment dissolve more completely.

6- Then, an ELISA reader device (ELx 808, BioTek, USA) operating at a wavelength of 570 nm was used to determine the concentration of the chemical dissolved in isopropanol.

7- In comparison to the well with fewer cells, the well with more cells has a higher optical density (OD). Therefore, it can be determined from the relationship below the well with more cells and compared with the control sample (DMEM culture medium containing 10% FBS).

8- Statistical analysis was performed to calculate the IC_{50} (https://www.aatbio.com/tools/ic50-calculator), through the following equation:

Cell viability% =A(Test)/ A(Control) × 100

Cell inhibition = 100 - A(Test)/ A(Control) × 100

Cell death will Cause inhibition of biochemical reaction and formation of purple formazan. Biochemical enzymatic cascade in live cells will trigger reduction of substrate and will cause to change to purple wells color (Positive control).

3.2.17. Statistical analysis

The data values are presented as the mean ± S.D. Differences in mean values were analyzed by two-way ANOVA followed LSD test with the IBM SPSS Statistics version 27 software (International Business Machines Corp., Armonk, NY, USA). Values with a $P < 0.05$ were considered to indicate statistical significance (Daniel and Cross , 2018).

Chapter Four
Results and Discussion

4. Results and Discussion

4.1.Leeches Feeding and Saliva Collection

The effectiveness of antibiotics used to treat infections has decreased or vanished due to increased antibiotic resistance (Nwobodo *et al*., 2022). Each year, millions of people develop antibiotic resistance to a variety of illnesses, and tens of thousands of people die directly from such diseases (Uddin *et al*., 2021). Since humans are continually exposed to germs, it is crucial to look for novel antibacterial compounds in natural goods. As leeches that feed on human blood create multiple antibacterial compounds that are excellent antimicrobials, invertebrates in nature have techniques to live and digest various types of food (Sanchez *et al*., 2022).

Due to the production of chemicals in its saliva as a result of its innate immune defense system, which is generated from its feeding process and surviving strategies in its habitat, *Hirudo medicinalis* is one of the species that is most frequently used as a model in medicine. More than 100 chemicals are secreted by this organism, although only a small number of them exhibit any active qualities, such as anti-inflammatory, antibacterial, analgesic, and anticoagulant activities (Sig *et al*., 2017).

Destabilase, chloromycetin, theromacin, theromyzin, and peptide B, antimicrobial peptides (AMPs) have been discovered in leeches, however, studies on salivary proteomics and transcriptome have revealed more peptides with antibacterial effects (Baker and Macagno, 2017; Grafskaia *et al*., 2020).

The current work demonstrates that when leeches were brought in close proximity to the parafilm membrane after twelve weeks of starvation, they began drinking the phagostimulatory fluid. The best solution to feed leeches is saline 0.15 M + Arginine 0.001 M, leeches still suck the solution. This solution was highly tolerated by leeches, this result is agree with (Alaama *et al*., (2011) who revealed that after a 12-week period of starvation, the concentration peaked. After making several unsuccessful attempts, he discovered that it is much simpler to collect the saliva by immersing the leeches in an ice bath inside a plastic test tube, the freezing temperature causes the animal to regurgitate (vomit) the desired sucked solution and impairs its movement, which in turn makes it easier to squeeze in order to maximize the amount of saliva obtained. Last but not least, the animal is not killed by the ice; all that is required is for him to be placed back into warm water so that he can resume his normal activities and continue to exist for an indefinite amount of time, Only clear uncolored liquid was collected and put in micro tube containers (Alaama *et al*., 2011).

A method is described for obtaining dilute *Hirudo medicinalis* saliva by feeding leeches through a membrane on arginine/saline and squeezing them immediately after from the posterior end forwards. The process can be repeated at intervals. Yields are considerably higher than from salivary gland extracts (Rigbi *et al*.,1987).

Fatty acids are important constituents and commonly possess antimicrobial activities. In the present study twenty leeches prepared for crude leech oil extraction. The oil was obtained by using vacuum rotary evaporated apparatus, until all the diethyl ether was completely evaporated, leaving the absolute essential oil and stored in a glass flask at 22^{o}C (AL-Jumilly *et al*., 2017).

4.2.Antibacterial Activities of Crude Leech Salivary Extract and Oil Leech Extraction on Test Isolates

Table (4.1) showed antibacterial activity of test isolates against the undiluted crude leech salivary extract and crude leech oil extract were antibacterial. All of the test isolates' could not be inhibited by the crude leech salivary and oil extract. Standard antibiotics Amikacin ($_{30}$ µg) used as the positive control inhibited the growth of the test isolates at zones of inhibition of 25.00±4.1 mm, and 22.00±4.7 mm against *S. aureus* and *P. aeruginosa* respectively and Am ($_{10}$ µg) with inhibition zone 25±5.8 mm for *S. pyogenes*, and 23.00±4.4 mm for *E. coli* respectively.

While no inhibition was seen against any of the test bacteria in the present study, the Malaysian leech saliva extract demonstrated broad spectrum antibacterial activity against both Gram-positive (*S. aureus*) and Gram-negative (*S. typhi* and *E. coli*) bacterial strains. Theromacin and Theromyzin, on the other hand, were isolated from the leech. According to (Tasiemski, 2008), *Theromyzon tessulatum* exhibited inhibitory effect directly against Gram-positive rather than Gram-negative spp., indicating that the active peptides in Malaysian leech saliva extract may be a distinct type. Whereas the current study results showed that test isolates are not inhibited by crude leech saliva extract Figure (4.1) and (4.2).

Table (4.1): Antibacterial Activity of Crude Leech Salivary Extract and Leech Oil Extraction compared with Amikacin $_{30\ \mu g}$ and Ampicillin $_{10\ \mu g}$ on Test Isolates (ATCC)

Test Isolates	*Staphylococcus aureus* AN_{30}	*Streptococcus pyogenes* Am_{10}	*Escherichia coli* Am_{10}	*Pseudomonas aeruginosa* AN_{30}
Mean± SD Inhibition Zones of Antibiotic AN_{30} and Am_{10} on Test Isolates in mm	25.00±4.100	25±5.800	23.00±4.400	22.00±4.700
Mean± SD Inhibition Zones of Crude Leech Saliva and Crude Leech Oil Extract on Test Isolates in mm	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000

- **Values that differ significantly at ($P \geq 0.05$).**
- **Antibiotics discs Amikacin$_{30}$µg(AN_{30}) and Ampicillin$_{10}$µg(Am_{10})**

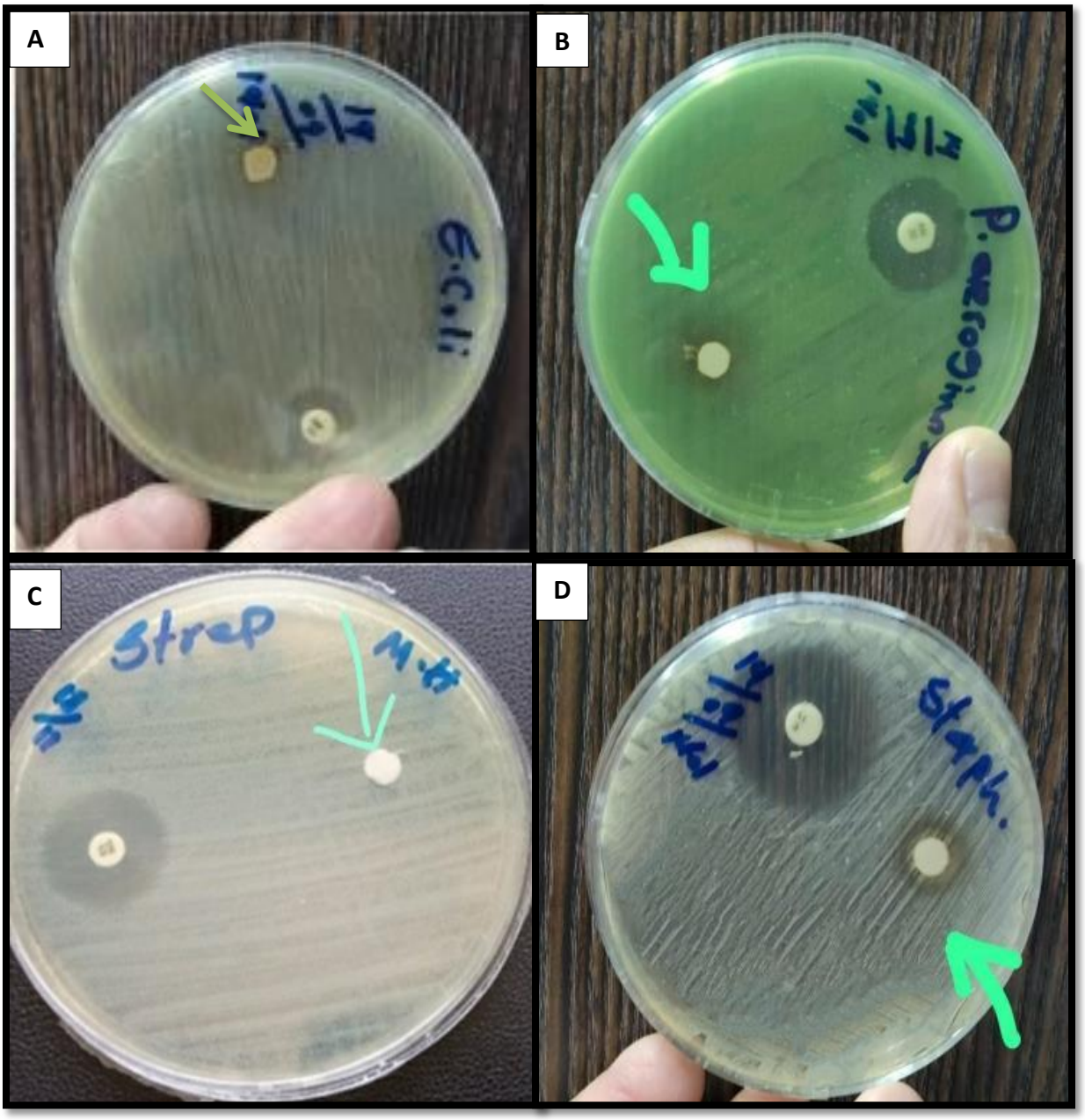


Figure (4.1): Negative antibacterial activity of crude *Hirudo medicinalis* saliva on ATCC bacterial strains (A) *E. coli* (B *Pseudomonas aeruginosa* (C) *Streptococcus pyogenes* (D) *Staphylococcus aureus* compared with positive control(Amikacin and Ampicillin). The pointer shows the negative antibacterial effect of crude leech salivary extract on MHA at 37 o C for 24 hrs.

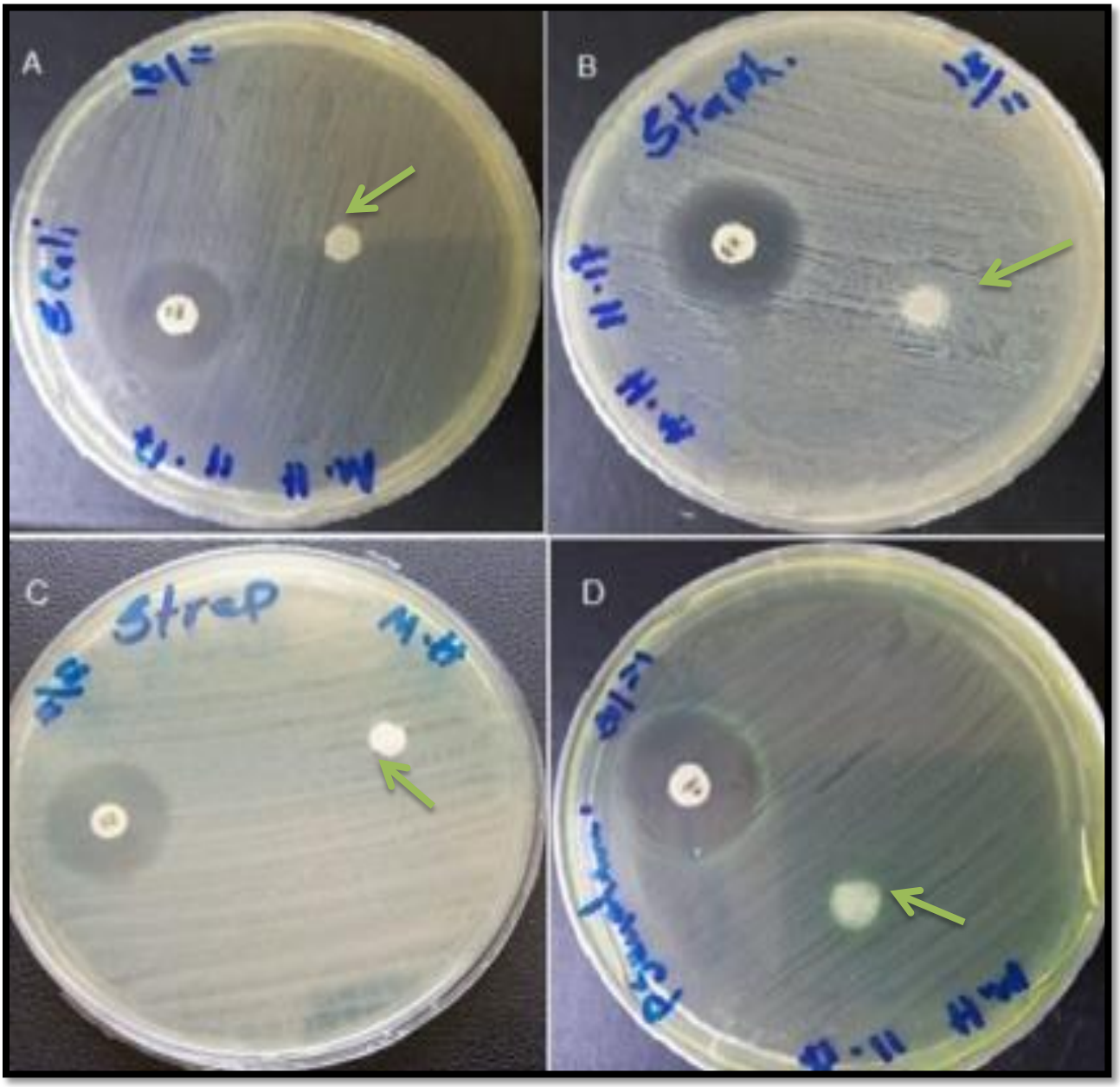


Figure (4.2): Negative antibacterial activity of crude *Hirudo medicinalis* oil extract on ATCC bacterial strains **(A *Escherichia coli* (B) *S. aureus* (C) *S. pyogenes* (D) *P. auroginosa*** compared with positive control(Amikacin and Ampicillin). The pointer shows the negative antibacterial effect of crude leech oil extract on MHA at 37 o C for 24 hrs.

One study show the leech oil extracts possessed antibacterial activities against a total of 7 bacteria (3 gram positive and 4 gram negative, the mean zone of inhibition ranged between 2.6 and 22 mm (AL-Jumilly *et al*., 2017). While in the present study antibacterial activity of crude leech oil extract showed negative results in the present study. The nature and level of antibacterial agents present in the extracts, and this mode of action on different test microorganisms may be contributed to the presence of a reaction between the oil extract and bacterial cell wall (AL-Jumilly *et al*., 2017). Low levels of bioactive compounds in the extract may be to blame for this (Ashraf and Bakri, 2018).

The author (Babayi *et al*., 2022) agrees who showed the crude leech salivary extract (CLSE) has no inhibitory effect on the test isolates. This result digresses with the findings of Malik *et al*., (2019), who claimed that several of the test isolates utilized in this study were inhibited by leech salivary extract. In which this study, CLSE was found to have an antimicrobial activity to the growth of *S. aureus.* This was different from what was found in the study of Malaysian researchers. The fresh leech saliva extract of the current study did not show any inhibition zone to the growth of these bacteria. This difference might be caused by the different conditions of the environment from where the leeches originated. The ecosystem where the leeches originally lived may have had distinct conditions, which could account for this inconsistency (Babayi *et al*., 2022).

4.3.Characteristics of Leech Salivary Extract- Mediated Silver Nanoparticles

Silver nanoparticles synthesized by leech salivary extract are linked to a shift in colorless solution to brown that lasts for 48 hours.

Silver nitrite were prepared in different final concentrations as follows:

(0.004, 0.006, 0.1, 0.6 mM). Only two from four concentrations of $AgNO_3$ (0.1, 0.6mM) show the change to brown color figure(4.3 A,B).

Figure (4.3A): Concentrations of $AgNo_3$ are(0.004, 0.006, 0.1, 0.6 mM) from left to right respectively at 37 ° C , 48 hrs. in aerobic condition .

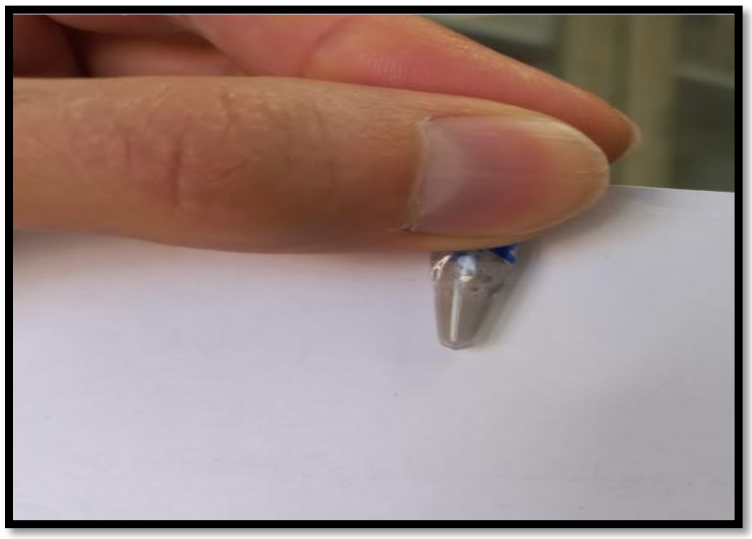


Figure (4.3B) After Nanoparticle formation color change to a brown due to oxidation reaction.

A color change from the colorless to dark brown was observed during the production of silver nanoparticles utilizing CLSE. Similar to this (Saravana *et al*., 2018) observed that surface Plasmon resonance excitation caused silver nanoparticles to dramatically change color in aqueous solution from colorless to dark brown. Because of Surface Plasmon resonance, the reaction mixture color changed from yellow to red . Those findings are similar to the results of (Abbas *et al*., 2021 ; Ullah *et al*., 2021) .

From available reports, these potentially active compounds have been elucidated as reducing and stabilizing agents (Khanna *et al*., 2019). According to related investigations, the reaction's color change from light yellow to brown suggested the reduction of silver ions to silver atoms (Badar and Khan, 2020).

The high technique known as field emission scanning electron microscopy (FE-SEM) is used to examine the topography of nanomaterials, including the size and form of nanoparticles. The field emission scanning electron microscopy (FE-SEM) results showed that samples include particles that are evenly scattered and nearly square in shape. The Nanoparticles ranged in size from roughly 20 to 720 nm. Figure (4.4) and Figure (4.5) images were captured a10 KX (10.000 x) and 35 KX, respectively, with an average value of 600 nm. 95% confidence was used when conducting the test.

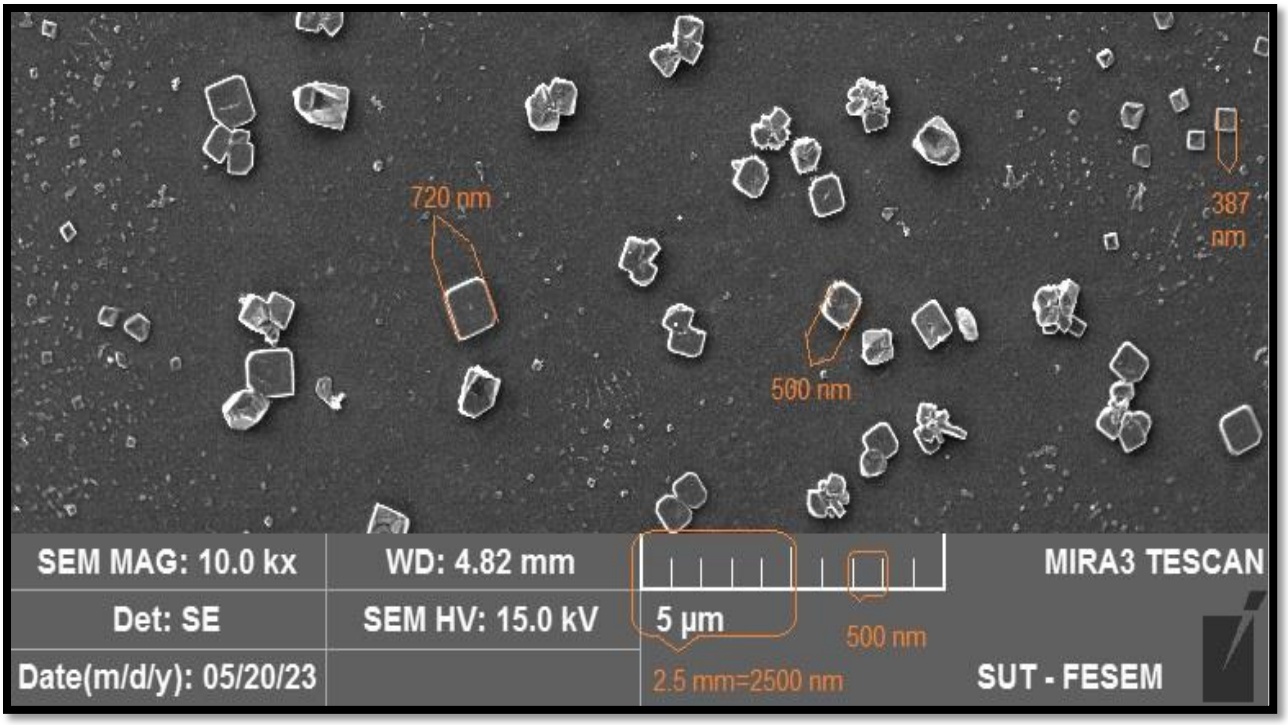


Figure (4.4) : FESEM images of the synthesized particles using Leech salivary extract at a magnification of 10 KX (10000 x).

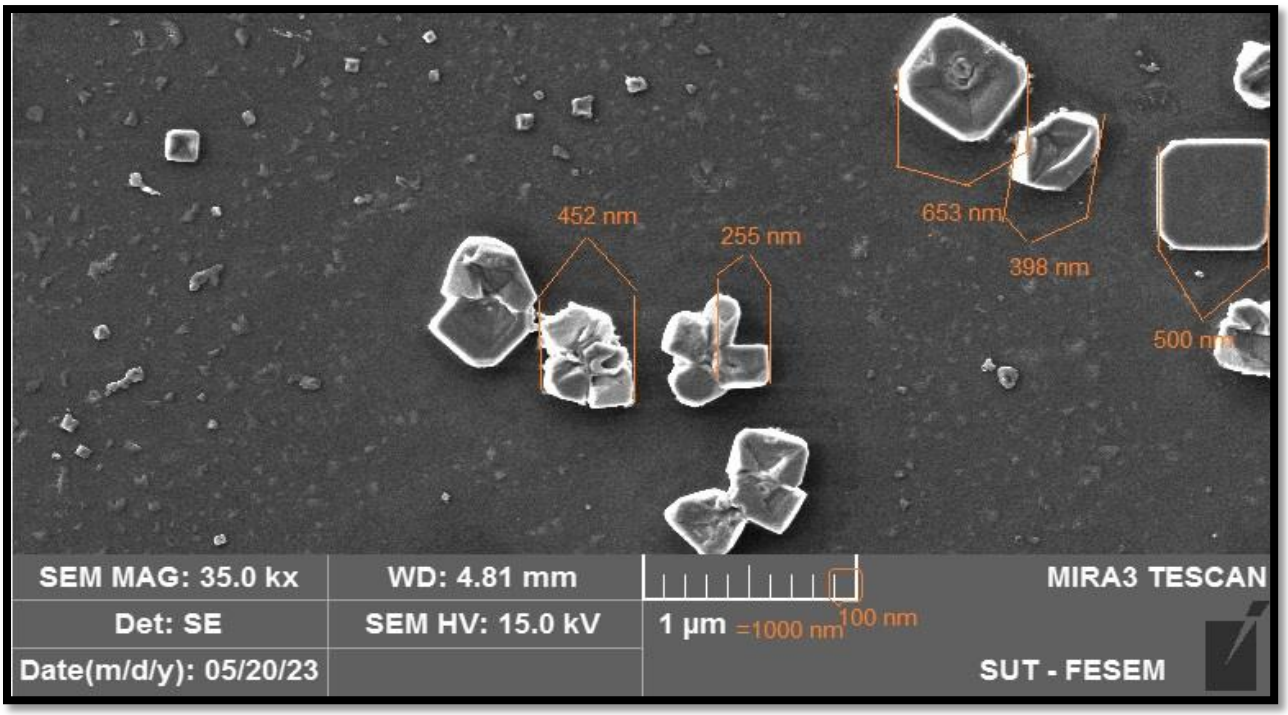


Figure (4.5) : FESEM images of the synthesized particles using Leech salivary extract at a magnification of 35 KX (35000 x).

Accepted results were obtained from (0.1mM) compared with the second concentrations (0.6mM) that show large size of nanoparticles and particles between (90-890nm), So we select the first one for further analysis. (0.1mM) of AgNo3 nanoparticles found in leech saliva have an average size of 649.1 nm and a zeta potential of -0.060 Appendix (1) and (2).

We are aware that a nanoparticle with a strong zeta potential (high negative or positive electrical charge) and a smaller size is more appropriate. Purity is just one of many variables that might affect a nanoparticle's characteristics. Leech saliva is not pure when it is extracted and contains a variety of substances, including several enzymes. They came to the conclusion that multi-leech therapy (MLT) is a reliable, conventional treatment with strong biochemical effects. Even though their mechanisms of action and bioactive materials are still being studied, these substances have clear use in specific medical situations. In order to further cancer research and leech therapy, efforts must be undertaken to extract salivary proteins and formulate them in Nano-carriers (Shakouri and Wollina, 2021). These impurity compounds may be added to the surface of nanoparticles during their synthesis, which can lead to big size and a weak zeta potential, as was the case in our work.

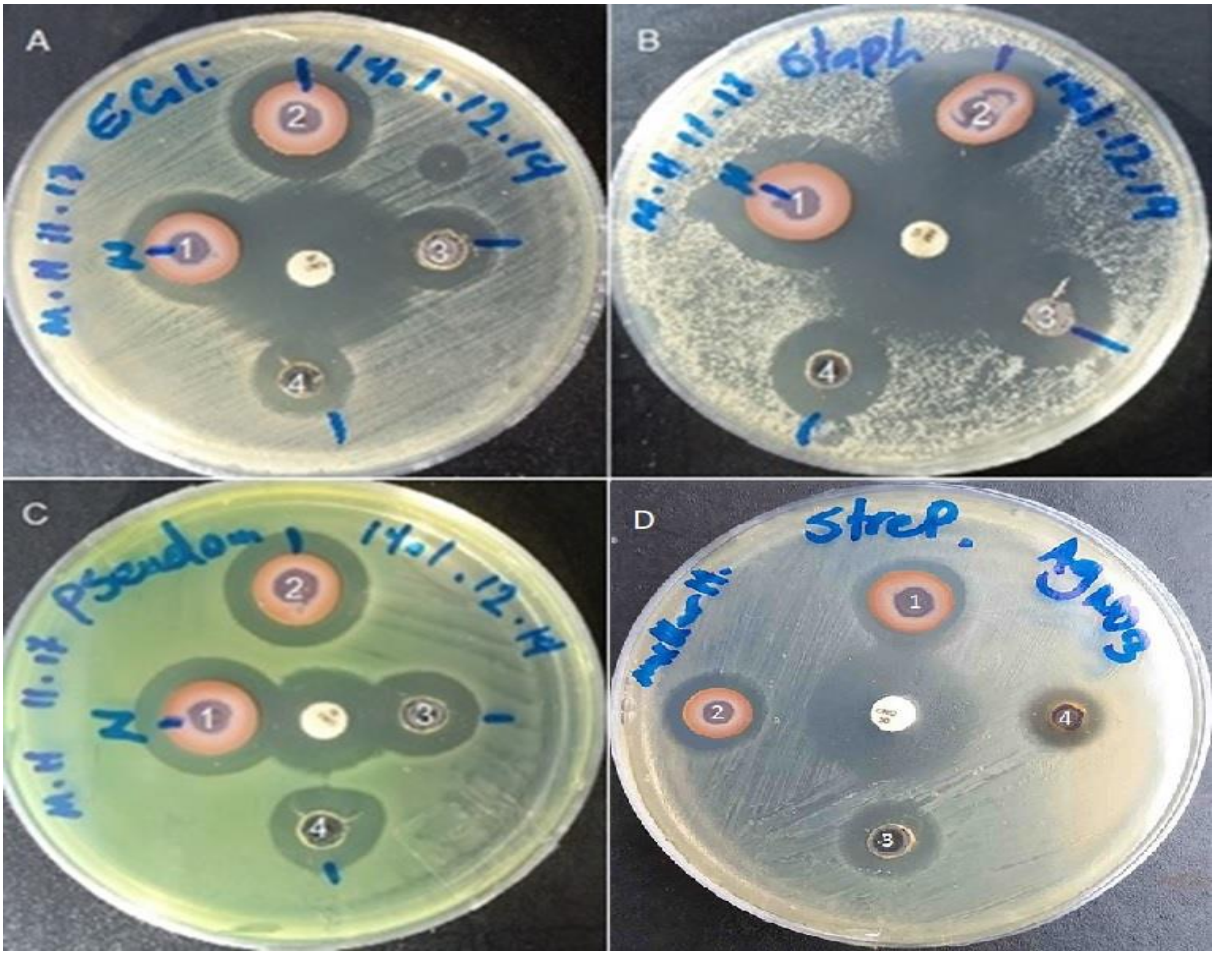
According to (Saeb *et al*., 2014) AgNPs are primarily considered stable when their surface charge is greater than +30 mV or less than -30 mV, based on the zeta potential of the nanoparticles. considering that the repellent interactions between the nanoparticles will stop them from aggregating. Both the coating agent and the synthesis method may specify this parameter (Chen *et al.,* 2016).

Leech salivary extract was used to synthesis the nanoparticles, as seen in FESEM photos. Protein, vitamins, amino acids, and antioxidants found in abundance in leech saliva have been crucial lowering agents of leech saliva-AgNP (Shakouri and Wollina, 2021). This agree with (Rajendran *et al*., 2015) in which from the FE-SEM image the size of the $AgNO_3$ obtained was greater than 1000 nm size and (Badar and Khan, 2020). SEM pictures are displayed at a size scale of 500 and 100 nm, respectively. while this result is not agree with (Babayi *et al*., 2022) who revealed that the size of nanoparticles from leech saliva was 98 nm. More active species mean a faster rate of nucleation and less time for the nucleated NPs to attach to the capping agents, which stop the process from continuing. The size and quantity of produced particles increase due to the quicker rate of reaction (Kumar *et al*., 2010). The size of NPs is influenced by both the rate of reaction and the contribution of surfactants (Song and Kim, 2009). When $AgNO_3$ is converted to Ag in the presence of capping agents, the nucleation process is maintained. The quantity of surfactants/capping agents and the rate of decreases control how evenly the particles are dispersed. The size of synthesized NPs is bigger when there are less surfactants and more reducing molecules present in the reaction mixture (Nune *et al*., 2009).

4.4.Antibacterial Activities of Leech Salivary Extract-Mediated Silver Nanoparticles on Test Isolates

Figure (4.6), table (4.2) display the antibacterial activity of silver nanoparticles mediated by leech salivary extract against four isolates *S. aureus, S. pyogenes, E. coli* and *P. auroginosa* at (15 µl). LSE-AgNPs with different concentrations (100, 200, 300, 400 µg/ml) showed inhibition activities against all

tested bacteria. The highest inhibition zone of LSE-AgNPs observed in concentration 400 µg/ml, while the lower inhibition zone observed in concentration 100 µg/ml .This inhibitory effect increased when the LSE-AgNPs concentrations were increased from 100µg to 400µg. CRO_{30}µg used as positive control for *E. coli* and *P. auroginosa*, and Am_{10} µg as positive control for *S. aureus* as positive control for *S. pyogenes*.



Figure(4.6): LSE-AgNPs Antibiogram. , A) *Escherichia. Coli,* B) *Staphylococcus aureus,* C) *Pseudomonas auroginosa* and D) *Streptococcus pyogense,* Nanoparticle concentration in each well is 1) 100, 2) 200, 3) 300, 4) 400 µg/ml Ceftriaxone and Ampicillin as positive control on MHA at 37 o C for 24 hrs.

Table (4.2) : Diameter of Inhibition zone of LSE-AgNPs against four ATCC isolates

Bacteria	Inhibition Zone (mm)				
	100µg/ml	**200µg/ml**	**300µg/ml**	**400µg/ml**	**Antibiotics**
S. aureus	**18**	**23**	**26**	**28**	**35 (Am10 µg)**
S. pyogenes	**24**	**26**	**28**	**29**	**25 Am10 µg)**
E. coli	**15**	**18**	**22**	**24**	**30 (CRO30 µg)**
P.auroginosa	**16**	**19**	**21**	**22**	**20 (CRO30 µg)**

- **Am= Ampicillin 10 µg**
- **CRO= Ceftriaxone 30 µg**

According to the last antibiogram results, we found that the suitable concentration of nanoparticle is 100 µg/ml. Therefore, in order to determine the Minimum Inhibitory Concentration (MIC) of bacteria, serial dilutions were prepared.

Numerous investigations have revealed that the AgNPs damage DNA, induce oxidative stress, alter membranes, and malfunction proteins in bacteria, all of which eventually result in bacterial death. AgNPs have also been observed to modify bacterial cell adhesion in order to inhibit the formation of biofilms (More *et al*., 2023).

Currently, the research mostly supports three methods (Marambio-Jones and Hoek, 2010; Daka *et al*., 2016; Qing *et al*., 2018) through which Ag-NPs exert their antibacterial function. According to the first theory, Ag-NPs affect cells at the membrane level because they can pass through the outer membrane and accumulate in the inner membrane, where their

adhesion causes the cell to become damaged and destabilized, increasing membrane permeability and leading to cell death (Seong and Lee, 2017; Ivask *et al*., 2014).

Staphylococcus aureus, *Escherichia coli*, *Streptococcus pyogenes* and *Pseudomonas auroginosa* growth were reported to be inhibited by (15µl) of leech salivary extract-mediated silver nanoparticles (CLS-AgNPs) at 100, 200, 300 and 400µg/ml. Although we agree with (Jaganathan *et al.* 2016) who utilized (10µl) of Earth Worm-AgNP in this test, crude leech salivary extract mediated nanoparticles was evaluated for antibacterial activity on the test isolates at 100 µl and 200 µl , respectively, using the agar well diffusion technique according to Babayi *et al*., (2022).

Results of current study of MIC and MBC for four bacterial isolates show the diameter of the inhibitory zone of (10 µl) AgNPs from high to low concentrations, accordingly, in comparison to positive and negative control. The results reveal that there are significant differences with different concentrations of AgNPs for each bacteria, but there are no significant differences between the 100µg/ml, 50µg/ml concentrations of AgNPs, but significant differences compared with other concentrations and antibiotics at $p \leq 0.05$ for all bacterial isolates.

One study demonstrated the various synthesis techniques and how they affect Multi Drug Resistant (MDR) bacterial infections. The activity is caused by the NP's internalization and interactions with intracellular content if the particles are between 10 and 20 nm in size. However, the action is caused by the silver ions when the size of the NPs exceeds 20 nm (More *et al*., 2023).

4.5.Minimum Inhibitory and Minimum Bactericidal Concentrations of Leech Salivary Extract-Mediated Silver Nanoparticles on Test Isolates

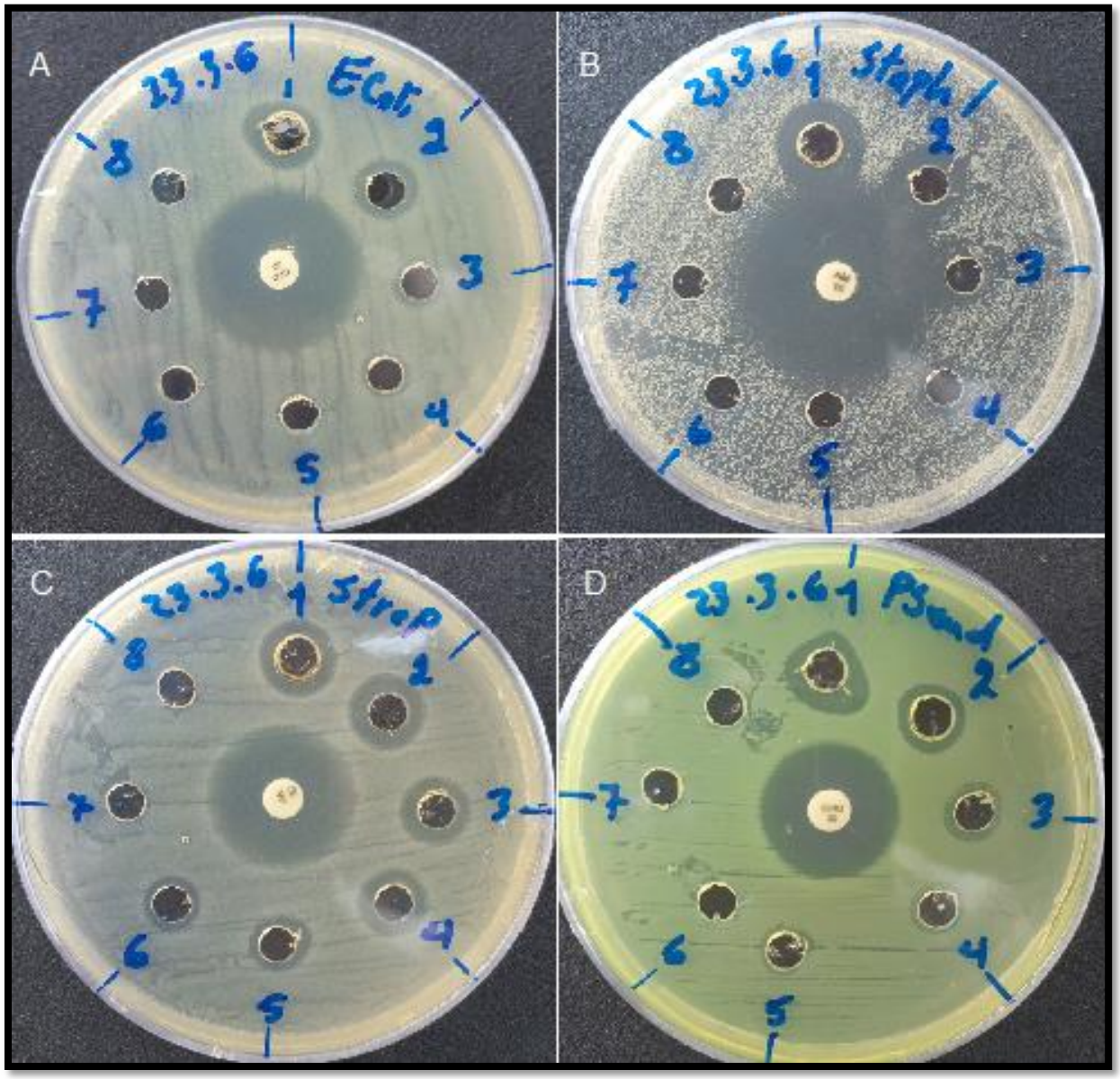
Table (4.3) and figure (4.7) revealed size of inhibition zone of AgNPs (15µl of 100 µg/ml), from high concentration 100 µg/ml to low concentration 3.13 µg/ml respectively in mm for S. aureus , S. pyogenes, E. coli and P. aeruginosa compare to antibiotics and negative control (PBS). The results revealed that there are significant differences with different concentrations of AgNPs for each bacteria, no significant differences between the 100µg/ml and 50µg/ml concentrations of AgNPs, but significant differences compared with other concentrations and antibiotics at $p \leq 0.05$ for all bacterial isolates.

Table(4.3): Size of inhibition zone of LSE-AgNPs from high to low concentration respectively in mm for *S. aureus , S. pyogenes, E. coli and P. aeruginosa* compare with positive control (antibiotics) and negative control (PBS).

AgNPs con. (µg/ml) / Bacteria	Inhibition zone in mm								LSD(0.05)
	100	50	25	12.5	6.25	3.13	Antibiotics	PBS	
	Mean± S.D								
S.aureus	26±1.9 d	26±1.9 d	21±0.9 c	16±2.2 b	0±0 a	0±0 a	AM10 35±3.9 e	0±0 a	1.247
Str.pyogenes	27±2.5 d	27±2.5 d	27±2.5 d	20±1.3 c	15±1.4 b	10±1.1 b	AM10 25±5.8 d	0±0 a	2.163
E.coli	22±3.1 c	22±3.1 c	15±1.4 b	15±1.4 b	0±0 a	0±0 a	CRO30 30±4.3 d	0±0 a	1.573
P.auroginosa	23±1.4 d	21±0.9 c d	16±2.2 b	16±2.2 b	0±0 a	0±0 a	CRO30 20±1.3 c	0±0 a	2.350

- Values are Mean± S.D, different letters refer to significant value at $p \leq 0.05$.
- LSD= least significant differences.

- Negative control: PBS buffer.
- AgNPs: silver nanoparticles from *H. Medicinalis*
- positive control= Am_{10}: Ampicillin(10µg), CRO_{30}= Ceftriaxone (30 µg)



Figure(4.7): Antibiotic Susbitibility Test (Agar Well Diffusion) A) ***E. coli*, B) *S .aureus*, C) *S. pyogenes*, D) *P. aeruginosa*** compared to positive control antibiotics($Am_{10µg,}$ $CRO_{30µg}$) and negative control (PBS) on MHA at 37 o C for 24 hrs.

Serial dilutions in all plate are equal and include: Well 1; 100µg/ml as Positive control, well 2; 50µg, well 3; 25µg/ml, well 4; 12.5µg/ml, well 5; 6.25/ml, well 6; 3.13µg/ml, well 7; Crude saliva, well 8; Negative control. Proportional standard sensitive antibiotic for each bacterium is, *E. coli* and *P.auroginosa* Ceftriaxone (CRO_{30}µg), *S.aureus* and *S.pyogenes* Ampicillin (Am_{10}µg).

Research suggested that variations in the composition of the cell walls of Gram-positive and Gram-negative bacteria are linked to their differing levels of antibiotic activity. Because the cell walls of Gram-positive bacteria contain a higher amount of peptidoglycan, it has been found that AgNPs are less efficient against them. Gram-negative bacteria have a lipopolysaccharide coating that attracts the positive charge of AgNPs, making it difficult for AgNPs to kill Gram-positive bacterial cells. AgNPs are therefore shown to be far more effective against Gram-negative bacteria than they are against Gram-positive bacterial cells. The current results of the MIC and MBC effects of CLSE-AgNPs on four test isolates, which show that *S.pyogenes* is more susceptible than other isolates in both MIC and MBC tests, are not consistent with the findings of this study. In the case of microbes, the powerful mechanism of NPs is dependent upon a variety of physical parameters, including size, shape, and charge on molecules (Zhang *et al*., 2020).

According to study, this impact might result from AgNPs oxidizing in aqueous medium when exposed to air, a mechanism that lowers the particles' antibacterial potency (Lu *et al*.,2013).

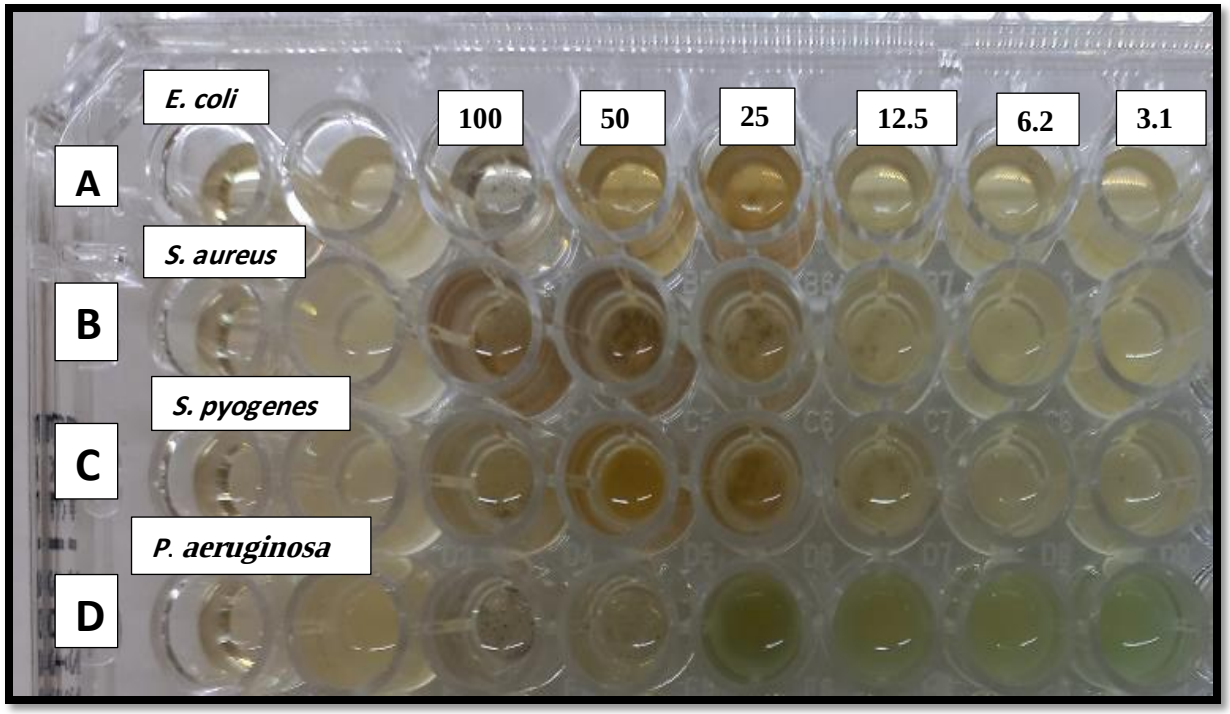
Table(4.4) reveal the minimum inhibitory and bactericidal concentration of leech salivary extract-mediated silver nanoparticles on test isolates. The MIC of the LSE-AgNPs against *E. coli* , *S. aureus* and *P. aeruginosa* were (50µg/ml) whereas the MBC of these bacteria were (100µg/ml) and the MIC of the LSE-

AgNPs against *S. pyogenes* was (25µg/ml) whereas the MBC was(50µg/ml) figure(4.8).

Table (4.4): Minimum Inhibitory and Bactericidal Concentration of Leech Salivary Extract Silver Nanoparticles on Test Isolates

Test Isolates	Concentrations of LSE-Ag-NPs (µg/ml)	
	MIC (µg/ml)	MBC (µg/ml)
Escherichia coli	50	100
Staphylococcus aureus	50	100
Streptococcus pyogenes	25	50
Pseudomonas aeruginosa	50	100

- **LSE-AgNPs (µg/ml)**: silver nanoparticles mediated *H. medicinalis* saliva at (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.13µg/ml) respectively.



Figure(4.8): MIC Test in Microtiter Plate. Rows A; *E. coli*, B; *S.aureus*, C; *S. pyogenes*, D; *P.aeruginosa* in Microtiterplate. Columns 1; Negative control 100µl Mueller Hinton broth only, 2; Positive(growth)control, 3-8; serial dilution including 3=100µg, 4=50µg, 5=25µg, 6=12.5µg, 7=6.25µg, 8=3.13µg of LSE-AgNPs

In a different study, the same agents and general procedure were used to synthesis AgNPs of various sizes, but the pH and the ratios of reducing and stabilizing agents in the reactions were altered. Subsequently, (Agnihotri *et al*., 2014) assessed the bactericidal and bacteriostatic potential of nanoparticles ranging in size from 5 to 100 nm against both Gram-positive and Gram-negative bacteria. For strains of *E. coli, Bacillus subtilis, and S. aureus*, the MIC values ranged from 20 to 110, 60 to 160, 30 to 120, and 70 to 200 µg/ml respectivly the first value corresponded to the smaller nanoparticles (5 nm), while the second value was associated with the larger nanoparticles (100 nm). Furthermore, for every strain under investigation, bactericidal doses ranged from 30 to 140 µg/ml; nonetheless, *S. aureus* in which the MBC (minimum bactericidal concentration) exceeded

200µg/ml. The increased surface area of the smaller nanoparticles, which is available for direct interaction with the bacterial cell, is thought to be the reason for the significant dependence of the antibacterial activity on size, as demonstrated by the MIC values (Bruna *et al*., 2021).

The difference between the findings of different studies is mainly due to the difference in the size of nanoparticles and the type of bacteria used . One of the most important factors affecting the antibacterial properties of nanoparticles is the particle size and concentration. It was considered that smaller nanoparticles had increased the production of ROS than larger surface area to volume ratio inside or out of the cells (Van Khanh and Van Cu , 2019) .

The stability of the products generated is also a significant factor influencing the ultimate antibacterial activity in proportion to the size and charge of the nanoparticles (Chen *et al*., 2016). Low stability of the generated AgNPs will cause them to aggregate and form larger particles, and research has shown that larger nanoparticles have less antibacterial action. The charge and the coating are the main variables influencing AgNP stability (Bruna *et al*., 2021).

One study revealed that synthesis of silver nanoparticles from *S. fusiformis* against the different bacterium *E. coli* and *K. pneumonia* has a towering inhibition and got decreased gradually against *S. aureus* and *P. aeruginosa* respectively, whereas silver nanoparticles have more or less similar effect on the bacteria. The mode of the bactericidal activity of silver nanoparticle is depending on the source from which the particles derived (Karthick *et al*., 2011).

The silver nanoparticle has various modes of action when invaded to microbes. It depends on different parameters like source, concentration and contact time, nature of microbe, temperature and pH. The exact mechanisms of silver

nanoparticles against the bacterial culture are clearly known and the small surface area containing nanoparticles having interaction with the large surface area may attach the cell membrane of the bacteria and involve the process of upsetting the respiration and permeability. The adsorption on the bacterial surface and intracellular enzyme activity is the main reason for the antibacterial reactions (Murugesan *et al*.,2017).

Studies indicate that when bacteria were exposed to silver nanoparticles, changes in the membrane's morphology occurred that resulted in a significant increase in permeability, affecting proper transport through the plasma membrane. This rendered the bacterial cells unable to properly regulate transport through the plasma membrane, leading to cell death. The mechanism by which the nanoparticles are able to penetrate the bacteria is not fully understood (Supraja *et al*., 2015).

According to (Ojo *et al*., 2018), LSE's activity is caused by bioactive substances such proteins and antimicrobial peptides (AMP). The submicron (minute) size of silver nanoparticles, which allows them to act as a drug carrier by absorbing bioactive molecules (hydrophilic and hydrophobic) into their matrix, may be the cause of the observed activity in the CLSE-AgNPs as different to the crude LSE equivalent. By interacting with the thiol group of certain enzymes or respiratory enzymes, the bioactive compounds in the matrix of the LSE-Ag may limit the growth of bacteria, resulting in their demise according to studies by (Malik *et al*., 2019 ; Shikha *et al*., 2020).

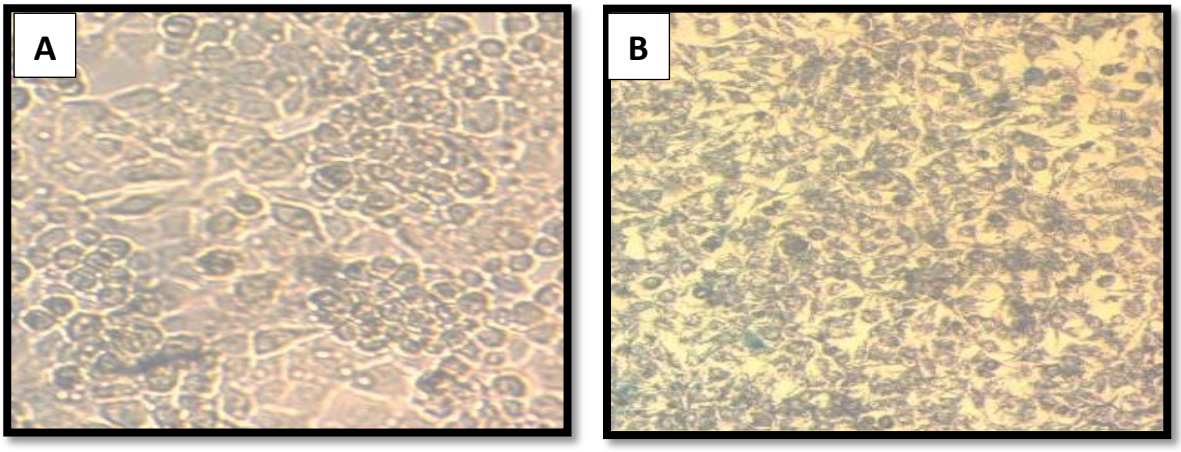
Denaturation and cell death are caused by the generation of reactive oxygen species, which attack microbial cells or aggregate in pits that form

on the cell wall and punch holes in the membrane of microorganisms. It has been noted that silver nanoparticles have entered the bacteria and have harmed them by interacting with DNA and other substances containing phosphorus and sulfur. The results of this work could influence the creation of novel antibacterial systems for use in medicine that are based on Ag-NPs (Supraja *et al*., 2016).

4.6. Quantitative Toxicity Test (MTT)

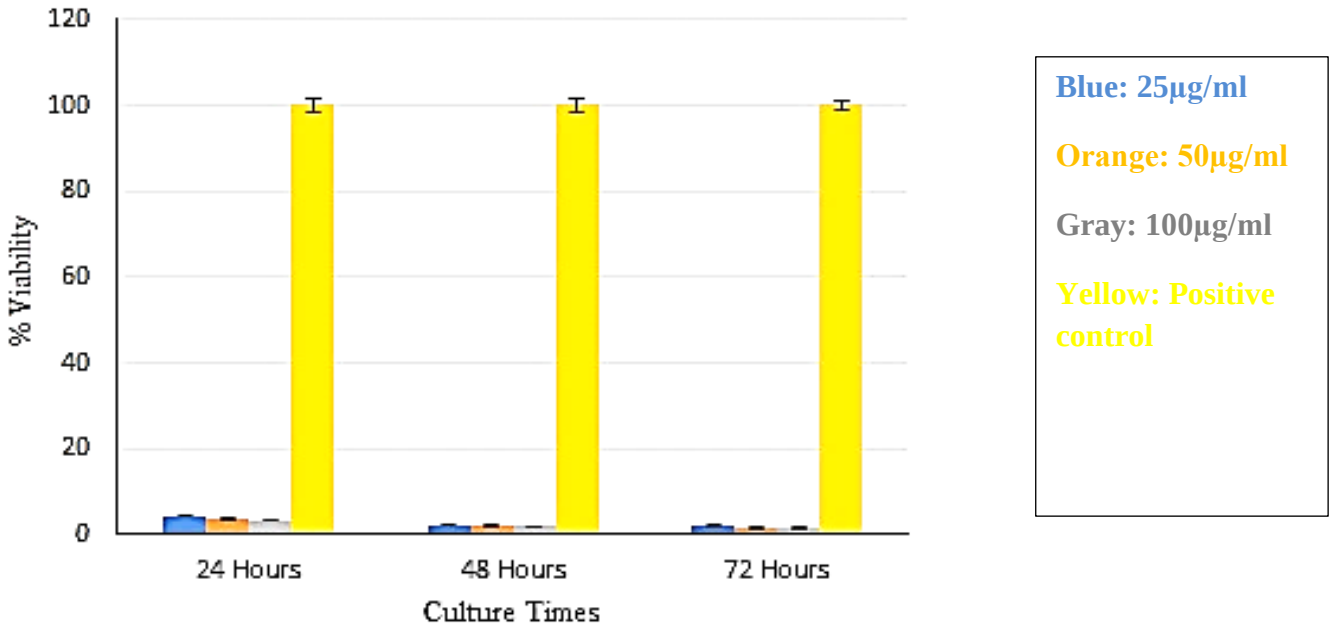
HepG2 cell coated in to the 96 well plate and contained with suitable culture medium (DMEM + 10% FBS). The purple crystals were dissolved with isopropanol after 100µl of MTT (M2128 Sigma Aldrich) with a concentration of 0.5 mg/ml was added to each well before being incubated for 4 hours solution had been in the cells Appendix(3).

Cell death will cause inhibition of biochemical reaction and formation of purple formazan. Biochemical enzymatic cascade in live cells will trigger reduction of substrate and will cause to change to purple wells color. (Positive control) figure(4.9A,B).



Figure(4.9): MTT assay: A: without treatment HepG2 cell line; B: Formazin precipitated HepG2 cell line

The *in-vitro* cytotoxic effects of leech saliva-$AgNo_3$ nanoparticle were screened against HepG2 cell line and the percentage of cell inhibition was confirmed by MTT assay. Toxicity test was performed in 3 determined nanoparticle concentration including 25, 50 and 100µg/ml association with 3 time follow-up including 24, 48 and 72 hours Figure(4.10) diagram of MTT test using HepG2 cell line and in presence of Leech saliva-$AgNo_3$ nanoparticle. The viability of HepG2 reduce after interaction with various concentrations of Ag NPs. This reduction increase gradually with the increase of the concentration and time of exposure to leech saliva-$AgNo_3$ nanoparticle. At 48 hours, this nanoparticle's IC_{50}(0.0124) value was 50µg /ml Table(4.5).



Figure(4.10): Diagram of MTT test using HepG2 cell line and in presence of Leech saliva-AgNo3 nanoparticle. **Blue: 25µg/ml, Orange: 50µg/ml, Gray: 100µg/ml, Yellow: Positive control**

Table(4.5): The effect of the exposure period and the concentrations of LSE-AgNPs (from *Hirudo medicinalis)* on the %viability of HepG2 cell line.

Period (hour)(B) / Conc.(A) LSE-AgNPs µg/ml	24	48	72
	Mean ±S.D of the viability% of HepG2 Cell Line		
Control	100±0.0	100±0.0	100±0.0
25	4.35±0.82%	2.14±0.13%	1.84±0.01%
50	3.57±0.14%	2.05±0.07%	1.51±0.13%
100	3.21±0.11%	1.80±0.02%	1.48±0.25%

- LSD($p \leq 0.05$)(A*B)=1.217
- LSE-AgNPs: Leech Saliva Extract Mediated Silver Nanoparticles.

According to one study (Mohammed *et al*., 2022), AgNPs exhibited lesser cytotoxicity toward normal cells (M-Stem cell and human fibroblasts (HF2), which showed that the viability of cells decreased at high doses of Nano-Ag, Additionally, a study that assessed the toxicity of AgNPs on human hepatocytes (Jabir *et al*., 2019; Kadhem *et al*., 2019) discovered that nano silver has hazardous effects on human hepatocytes at high concentrations but not at low concentrations. Another study by Plaza *et al*., (2014) discovered that high concentrations of AgNPs drastically reduced the viability of human gingival epithelial cells in in-vitro settings.

These results revealed the significant differences in the %viability among not treated cells (control HepG2 cell line) and treated cells with LSE-AgNPs, significant differences in the %viability among the cells that expose to the same concentration of LSE-AgNPs (25,50,100µg/ml) between (24 and 48hours) and (24

and 72hours), no significant differences among these cells between (48 and 72hours) and no significant differences among treated cells with different concentrations of LSE-AgNPs at same time.

Different silver nanoparticle manufacturing methods or particle sizes could account for the various hazardous amounts reported by earlier investigations. In our present study the IC_{50}(0.0124) of LSE- AgNPs concentrations was 50 µg/ml at 48 hrs. According to American National Cancer Institute recommendations, the limit of activity for crude extracts is less than 30 mg/ml for 50% inhibition (IC_{50}) of proliferation following a 24-hour exposure period (Suffness, and Pezzuto, 1990). Since LSE-AgNP's IC_{50} value of 50 µg/ml is below the conventional limit of activity, the extract from the leech that caught our attention has demonstrated its potential as a treatment for hepatocellular carcinoma. According to a different study, EW-AgNP's IC_{50} value of 25.96 µg/ml is within the accepted range of activity (Jaganathan *et al*., 2016).

The active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, nitrogen bases, and phosphate groups in DNA is what causes AgNPs to have lethal effects. However, more research is required to completely comprehend the mechanism underlying the anticancer effect. The healthy, grouped, and viable cells in the control group. According to (Supraja *et al*., 2016), three concentrations of silver nanoparticle treatment over three times resulted in somewhat greater morphological changes in the leech saliva silver nanoparticles treated cells. The clearly visible cell debris is caused by cell death. Further studies have to be carried out to understand the nature of cytotoxicity and

the death or proliferation of cells caused by *Gracilaria corticata* extract mediated silver nanoparticles (Mosmann,1983; Monks *et al*.,1991).

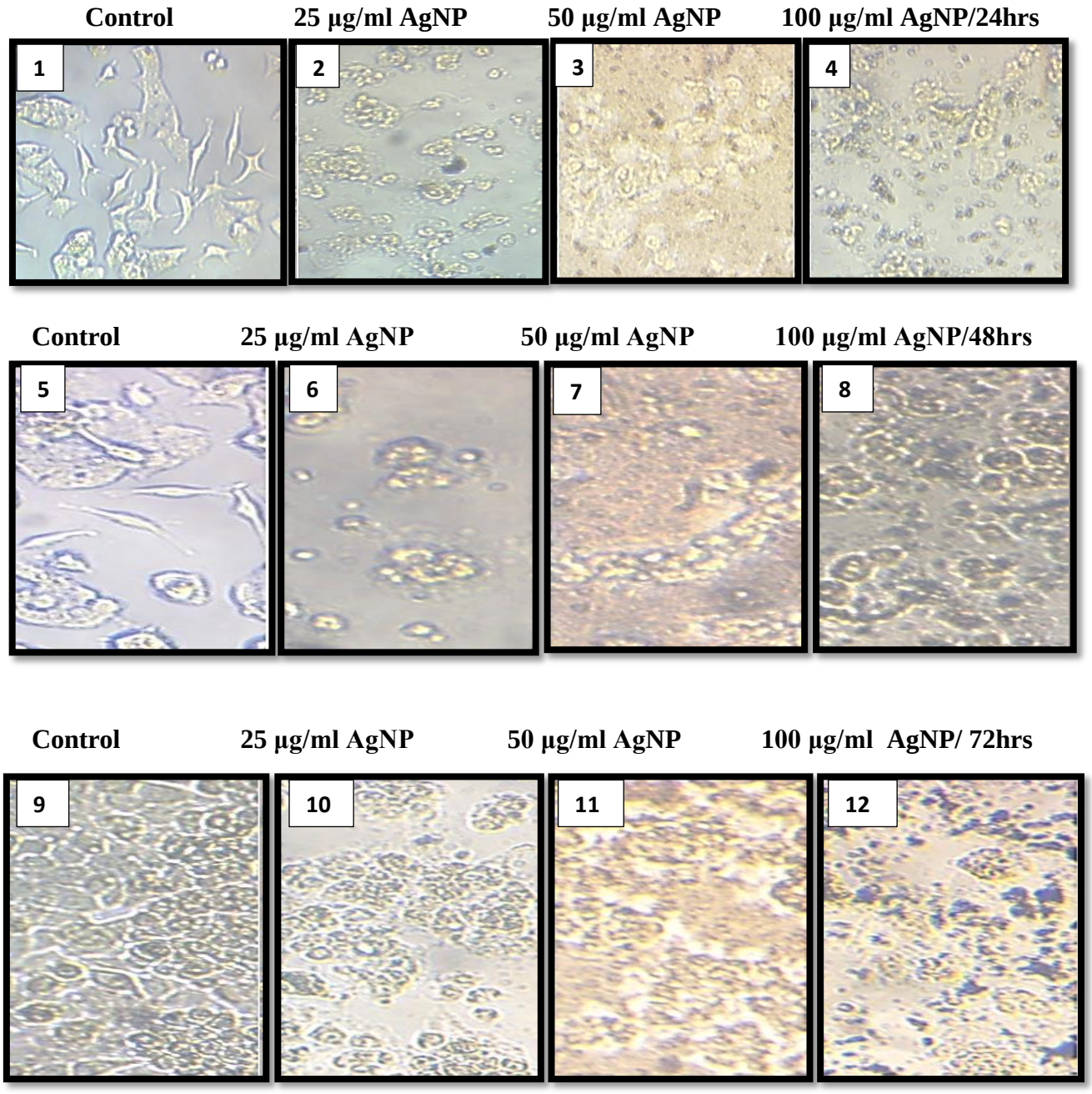
The process of naturally creating the silver nanoparticles from leech saliva extract proved to be both less effective and environmentally safe. Against a variety of bacterial species, these well- defined silver nanoparticles demonstrated improved antibacterial capabilities. According to earlier in vitro cytotoxic studies, AgNPs cause oxidative stress and induce apoptosis in human liver HepG2 cells as a result of mitochondrial injury through the production of reactive oxygen species or by increasing intracellular oxidative stress, which in turn causes cell death processes like apoptosis and necrosis (Rajeshkumar *et al*., 2016).

4.7. Morphological Changes in HepG2 Cells after AgNPs Exposure

The liver (HepG2) cancer cells was cultured and its morphology detail was observed via light microscopy after incubation for 24, 48 and 72 hrs. in the presence of varying concentrations (25, 50, and 100μg/ml) of AgNPs. Representative images of cell lines show remarkable changes in the morphology at AgNP concentrations of 25, 50, and 100μg/ml. The morphology of cells at 25, 50, and 100μg/ml between (24 and 48hours) was greatly affected

The toxic effect indicated by the viability assay may also reflect the abnormal aspect of the cell morphology. Cell morphology after exposure to Ag NPs was investigated using an optical microscope. When exposed to Ag NPs at concentrations of 25, 50 and 100 μg ml for 24, 48 and 72 hrs, the treated cells showed distinct morphological changes indicating unhealthy cells, whereas cells in the control group appeared to be normal. As the Ag NPs concentration and exposure time increase, the exposed cells appeared to be clustered with a few

cellular extensions, and cell spreading patterns were restricted with the observation of a few floating cells as compared with the control cells (Figure 4.11).



Figure(4.11): Morphological Changes in HepG2 Cells after CLS-AgNPs Leech saliva Exposure in MTT Test at three concentrations(100, 50,25 µg/ml) after 24 ,48 and 72 hrs. Untreated cells were included as controls. Microscopic images of HepG2 cells (treated and untreated). Magnification = 200×.

According to the MBC test results, different concentrations and times of prepared nanoparticles including 25, 50 and 100 µg/ml and 24, 48 and 72 hours respectively were investigated.1) HepG2 Control, 2) 25µg/ml 24 hrs. Test, 3) 50 µg/ml 24 hrs. Test, 4) 100µg/ml 24 hrs. Test 5) HepG2 Control, 6)25µg/ml 48 hrs. Test,7) 50µg/ml Test in 48hrs, 8) 100µg/ml Test in 48hrs, 9) Control, 10)25µg/ml test in 72 hrs., 11) 50µg/ml test in 72 hrs., 12) 100µg/ml test in 72 hrs.

One of the earlier studies (Vismara *et al*., 2013; Khan *et al*., 2019), the cytotoxicity of cancer cells is significantly impacted and is in charge of controlling cancer cell proliferation. Despite this, a number of other variables were also involved, including pH, cell composition, culture medium, dosages of nanostructures, and the shape of the nanostructures (Vismara *et al*., 2013; Steinmetz *et al*., 2020). The HepG2 lines were used to test the anticancer effect of silver nanoparticles, and the results indicate good cytotoxic activity against cancer cells. The amount of silver nanoparticles present has a significant impact on the anticancer activity. After 24, 48, and 72 hours, respectively, silver nanoparticles are showing promising outcomes, followed by 25, 50, and 100 µg/ml. After 72 hours, the lowest inhibitory activity was noted at a concentration of 100 µg/ml. According to our study, leech saliva may be used as a source for anti-cancer medications. Hepatocellular carcinoma (HepG2) cells were used as a test subject for the aqueous extract of the leech's saliva. It was discovered that the produced silver nanoparticles had anticancer properties and inhibit HepG2 cells in a dose-dependent manner. Some of the chemotherapy drugs that were authorized had expensive with side effects. Therefore, there is a critical need to create alternative medications to combat this fatal illness. A relatively easy and environmentally friendly

approach for the biosynthesis of silver nanoparticles utilizing leech saliva extract has been discovered. The production of AgNPs, which were efficient anticancer agents against HepG2 cancer cells, required the use of leech saliva, which contains proteins, as both a capping material and a reductant (Supraja *et al*., 2016).

The previously published literature reveals that most studies used either they higher concentrations of nanostructures or complex chemical compounds, which are not appropriate for human body (Pablo and Guy, 2004; Jeng and Swanson, 2006). From our experiments, we believe that a minute quantity of the nanostructure is sufficient to achieve the control of cancer cell growth. AgNPs have a very small size in solution, allowing them to more easily target cellular organelles compared with other complex and organic-based drugs.

According to the MTT and MBC assays, it was revealed that the inhibitory concentrations of LSE AgNPs for selected bacteria strains are toxic for HepG2 cell lines.

Conclusion and Recommendation

Conclusion

From the present study it is concluded

1- The crude leech salivary extract (CLSE) and crude leech oil extract showed no inhibitory effect on the test isolates of the present study.
2- The investigation of the present study revealed the potential of silver nanoparticles obtained from leech saliva as a rich source of antibacterial agent.
3- If we wish to expand our project, we can try to make much more pure nanoparticles and purer leech saliva. Our CLSE-AgNPs, however, is bioactive and exhibits adequate antibacterial activity.
4- The Leech salivary extract-mediated silver nanoparticles (CLSE-Ag) were observed to inhibit the growth of (ATCC) isolates *P. aeruginosa* , *E.coli* , *S. aureus* and *S. pyogenes*.
5- According to the antibiogram results, we found that the suitable concentration of crude saliva nanoparticle is 100µg/ml.
6- According MBC assays, it was revealed that CLSE- AgNPs have inhibitory effect for selected bacterial strains.
7- According to the MTT CLSE- AgNPs was toxic for HepG2 cell lines.

Recommendation

1- Biosynthesis of different metal nanoparticles by leech saliva and study its effect on bacteria.

2- Study the effect of leech saliva and leech oil on other microorganisms other than bacteria.

3- Use leech saliva and leech oil from different regions in the world for antibacterial study.

4- Study the activity of leech saliva against MDR bacteria.

5- Study the cytotoxic effect of CLSE-AgNPs on hepatocyte human cell line.

References

* Abbas, H. S., Abou Baker, D. H., and Ahmed, E. A. (2021). Cytotoxicity and antimicrobial efficiency of selenium nanoparticles biosynthesized by Spirulina platensis . *Archives of Microbiology*. 203(2): 523-532.

* Abdelghany, T.M.; Al-Rajhi, A.M.H.; Al Abboud, M.A.; Alawlaqi, M.M.; Ganash Magdah, A.; Helmy, E.A.M., and Mabrouk, A.S. (2018). Recent Advances in Green Synthesis of Silver Nanoparticles and Their Applications: About Future Directions. A Review. *BioNano Science*. 8: 5–16.

* Abdisa, T. (2018). Therapeutic importance of leech and impact of leech in domestic animals. *MOJ Drug Design Development and Therapy*, 2(6):235–242.

* Abdualkader, A.M.; Merzouk, A.; Ghawi, A.M., and Alaama, M.(2011). Some biological activities of Malaysian leech saliva extract. *IIUM Engineering Journal*.12(4):1–9.

* Abdualkader, A.M.; Ghawi, A.M.; Alaam, M.; Awang, M., and Merzouk, A. (2013). Leech therapeutic applications. Indian Journal of Pharmaceutical Sciences, 75(2): 127–137.

* Abdullah, S.; Dar, L.M.; Rashid, A., and Tewari, A.(2012). Hirudotherapy/leech therapy: applications and indications in surgery. *Archives of Clinical and Experimental Surgery.* 1(3):172-80.

* Agnihotri, S.; Mukherji, S., and Mukherji, S.(2013). Immobilized silver nanoparticles enhance contact killing and show highest efficacy: Elucidation of the mechanism of bactericidal action of silver. *Nanoscale Journal*. 5(16): 7328–7340.

*Agnihotri, S.; Mukherji, S., and Mukherji, S.(2014). Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy. *Royal Society of Chemistry Advance*. 4(8): 3974–3983.

* Ahirrao, R.; Jadhav, J., and Pawar, S. (2017). A review on leech therapy. *Pharma Science Monitor* 8(1): 228-237.

* Ahmad, S.; Munir, S.; Zeb, N,; Ullah, A.; Khan,B.; Ali, J.; Bilal,M.; Omer, M.; Alamzeb, M.; Salman, M., and Ali, S.(2019). Green nanotechnology: a review on green synthesis of silver nanoparticles – an ecofriendly approach. *International Journal of. Nanomedicine*. 14(10): 5087-5107.

* Ahmed, S.F.; Mofijur, M.; Rafa, N.; Chawdhury, A.T.; Chowdhury, S.; Nahrin, M.; Islam, A.B.M.S., and Ong, H.C. (2022). Green approaches in synthesising nanomaterials for environmental nanobioremediation: Technological advancements, applications, benefits and challenges. *Elsiver, Enviromental Research*. 204 part 1: 111967.

* Akhavan, A.; Sodagar, A.; Mojtahedzadeh, F., and Sodagar, K. (2013). Investigating the Effect of Incorporating Nanosilver/Nanohydroxyapatite Particles on the Shear Bond Strength of Orthodontic Adhesives. *Acta Odontologica Scandinavica*. 71(5):1038–1042.

* Akter, M.; Sikder, Md. T.; Rahman, Md.M.; Atique Ullah, A.K.M.; Hossain, K.F.B.; Banik, S.; Hosokawa, T.; Saito, T., and Kurasaki, M.(2017). A systematic review on silver nanoparticles-induced cytotoxicity: Physicochemical properties and perspectives. *Journal of Advanced Research*. 2(9):1-16.

* Alaama, M.; Alnajjar, M.; Abdualkader, A.M.; Mohammad, A., and Merzouk, A.(2011). Isolation and Analytical Characterization of Local Malaysian Leech Saliva. IIUM Engineering Journal. 12(4): 51-59.

* Albrecht, M. A.; Evans, C. W., and Raston, C. L., (2006). Green Chemistry and the Health Implications of Nanoparticles.*Green Chemistry journal*. 8(5): 417–432.

* Alessandro, N.D; Poma, P., and Montalto, G. (2007). Multifactorial nature of *hepatocellular carcinoma drug resistance could plant polyphenols be helpful? World Journal of Gastroenterology*. 13(14): 2037–2043.

* AL-Jumilly, E. F.; Montaha A.al-Safar, M. A., and Alubade, E. S.(2017). Determination of Minimal Inhibition Concentration and Minimal Bactericidal Concentration of Hirudo medicinalis oil on pathogenic bacteria. *Current Research in Microbiology and Biotechnology*. 5(5): 1273-1277.

* Alkhudhayri, A.A.; Wahab, R.; Siddiqui, M.A., and Ahmad, J.(2020**).** Selenium nanoparticles in-duce cytotoxicity and apoptosis in human breast cancer (MCF-7) and liver (HEPG2) cell lines. *Nanoscience* and *Nanotechnology Letters.* 12(1): 324–330.

* Ammar, A.E.; Hassona, M.H.; Meckling, G.R.; Chan, L.G.; Chin, M.Y.; Abdualkader, A.; Alaama, M.; Merzouk, A.; Helaluddin, A.B.; Ghawi, A.; Kucuk, O., and Guns, E. (2015). Assessment of the antitumor activity of leech (huridinaria manillensis) saliva extract in prostate cancer. *Cancer Research*. 75(15):5130.

* Anastas, P., and Eghbali, N.(2010). Green Chemistry: Principles and Practice. *Chemical Society Reviews*. 39(1):301-312

* Antony, J.J.; Sivalingam, P., and Chen, B.(2015). Toxicological Effects of Silver Nanoparticles. *Environmental Toxicology* and *Pharmacology* .40(3):729-32.

* Ashraf, A. M., and Bakri, M. M. (2018). Antimicrobial activity of some plants extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Science*. 25(2): 361-366.

* Atomsa, T.W. (2022). Virulence factor, pathogenesis and Laboratory diagnosis

technique of *Streptococcus pyogenes*: Review. I*nternational Journal of Advanced Research in Biological Sciences*. 9(8): 110-120.

***** Babayi, H.; Owolabi, B. I.; Adelere, I. A.; Mustapha, A.;A., and Amadi, E. D.(2022). Antibacterial Activity of Leech Salivary Extract- Mediated Silver Nanoparticles Against Some Pathogenic Bacteria. *Journal of Science, Technology, Mathematics and Education* (JOSTMED). 18(2): 25-34.

* Babenko, V.V.; Podgorny, O.V.; Manuvera, V.A.; Kasianov, A.S.; Manolov, A.I. Grafskaia, E.N.; Shirokov, D.A.; Kurdyumov, A.S.; Vinogradov, D.V.; Nikitina, A.S.; Kovalchuk, S.I.; Anikanov, N.A.; Butenko, I.O.; Pobeguts, O.V.; Matyushkina, D.S.; Rakitina, D.V.; Kostryukova, E.S.; Zgoda, V.G.; Baskova, I.P.; Trukhan, V.M.; Gelfand, M.S.; Govorun, V.M.; Helgi B. Schiöth, H.B., and Lazarev, V.N.(2020). Draft Genome Sequences of *Hirudo medicinalis* and Salivary Transcriptome of Three Closely Related Medicinal Leeches. *BMC Genomics*. 21(1):503

* Badar, W. , and Khan, M.A.U.(2020). Analytical study of biosynthesized silver nanoparticles against multi-drug resistant biofilm-forming pathogens. *IET Nanobiotechnol*. 14(4: 331-340.

* Bae, E.; Park, HJ.; Lee, J.; Kim ,Y.; Yoon, J., and Park, K. (2010). Bacterial cytotoxicity of the silver nanoparticle related to physicochemical metrics and agglomeration properties. *Environmental Toxicology* and *Chemistry*. 29(10): 2154-2160.

* Baker, M.W., and Macagno, E.R.(2017).Gap junction proteins and the wiring (Rewiring) of neuronal circuits. *Developmental Neurobiology*. 77(5):575-86.

* Baskova, I., and Zavalova, L.(2001). Proteinase inhibitors from the medicinal leech Hirudo medicinalis. *Biochemistry* (Moscow). 66(7):703-14.

* Baranowski C, Welsh MA, Sham LT, Eskandarian HA, Lim HC Kieser, K.J.; Wagner, J.C.; McKinney, J.D.; Georg E Fantner, G.E.; Thomas R Ioerger, T.R.; Walker, S.; Bernhardt, T.G. ; Rubin, E.J., and Rego, E.H. (2018). Maturing Mycobacterium smegmatis peptidoglycan requires non-canonical crosslinks to maintain shape. *Elife* 7: e37516.

* Baskova, I.P.; Kostrjukova, E.S.;Vlasova, M.A.; Kharitonova, O.V.;Levitskiy, S.A.; Zavalova, L.L.; Moshkovskii, S. A. and Lazarev, V. N .(2008). Proteins and peptides of the salivary gland secretion of medicinal leeches Hirudo verbana, H. medicinalis, and H. orientalis. Biochemistry (Mosc). 73(3):315-320.

* Baskova, I.P.; Zavalova, L.L.,;Basanova, A.V.; Moshkovskii, S.A., and Zgoda, V.G.(2004). Protein profiling of the medicinal leech salivary gland secretion by proteomic analytical methods. *Biochemistry* (Moscow). 69(7):770-775.

* Beer, C.; Foldbjerg, R.; Hayashi, Y.; Sutherland, D.S., and Autrup, H.(2012). Toxicity of Silver Nanoparticles—Nanoparticle or Silver Ion? *Toxicology Letters*. 208(3):286-292.

* Becanim, F.; Berktaş, S.A., and Ceylan, M.(2022). What is Need to Be Known About Medicinal Leeches and Hirudotherapy? A Comprehensive Review. *Anadolu Tıbbı Dergisi.* 1(3): 23 – 36.

* Bereda, G. (2022). Clinical Pharmacology of Ampicillin. *Journal of Pharmaceutical Research & Reports*. 3(3): 1-3.

* Blankenship, D.T.; Brankamp, R.G.; Manley, G.D., and Cardin, A.D. (1990). Amino acid sequence of ghilanten: Anticoagulant-antimetastatic principle of the South American leech, Haementeria ghilianii. *Biochemical and Biophysical Research Communications*.166(3):1384-1389.

* Block, M. and Blanchard, D.L. (2022). StatPearls [Internet]. *StatPearls Publishing; Treasure Island* (FL). Aminoglycosides.

* Bomar, L.; Maltz, M.; Colston, S., and Graf, J.(2011). Directed culturing of microorganisms using metatranscriptomics. *Microbiology* 2(2):e00012-11.

* Bruna, T.; Maldonado-Bravo, F.; Paul Jara, P., and Caro, N. (2021). Silver Nanoparticles and Their Antibacterial Applications. *International Journal of Molecular* Sciences. 22(13):1-21.

* Bush, K., and Bradford, P.A. (2016). β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harb Perspect Med*. 6(8): 1-22.

* Cardin, A.D.; Sunkara, S.P., and Munich.(1994). Ghilanten antimetastatic principle from the south American leech Haementeria ghilianii Germany: European Patent Office. *Indian Journal Pharmaceutical Sciences.* 75(2): 127–137.

* Carlson, C.; Hussain, S.M.; Schrand, A.M.; Braydich-Stolle, L.K.; Hess, K.L.; Jones, R.L., and Schlager, J.J.(2008). Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species. *Journal of Physical Chemistry* B. 112, 13608–13619.

* Celton-Morizur, S.; Merlen, G.; Couton, D., and Desdouets, C. (2010). Polyploidy and liver proliferation: central role of insulin signaling. *Cell Cycle*. 9 (3): 460–6.

* Chaloupka, K.; Malam, Y., and Seifalian, A.M. (2010). Nanosilver as a New Generation of Nanoproduct in Biomedical Applications. Trends, *Biotechnology* 28(11): 580–588.

* Chattu, V. K., and Yaya, S. (2020). Emerging infectious diseases and outbreaks: implications for women's reproductive health and rights in resource-poor settings. *Reproductive Health*, 17(1):43-51.

* Chen, J.; Li, S.; Luo, J.; Wang, R., and Ding, W. (2016). Enhancement of the Antibacterial Activity of Silver Nanoparticles against Phy- topathogenic Bacterium Ralstonia solanacearum by Stabilization. *Journal* of *Nanomaterials*. 2016: 1-15.

* Clinical and Laboratory Standards Institute, (2021). Performance Standard for Antimicrobial Susceptibility Testing; 3lst Edition. 41(3):22-42.

*Coyle, M.B. ; Lucas- G.L.R.; Ramirez- C.L.C., and Arregui, L.(2017). Manual of Antimicrobial Susceptibility Testing. American Society for Microbiology. *Advances in Biological Chemistry*. 7(2)p:10-13.

* Cortivo, R.; Vindigni, V.; Iacobellis, L.; Abatangelo, G.; Pinton, P., and Zavan, B. (2010). Nanoscale Particle Therapies for Wounds and Ulcers. *Nanomedicine*. 5(4):641-56.

* Dakal, T.C.; Kumar, A.; Majumdar, R.S., and Yadav, V.(2016). Mechanistic basis of antimicrobial actions of silver nanoparticles. Frontiers. *Microbiology*. 7:1831.

* Daniel, W. W., and Cross, C. L. (2018). *Biostatistics: a foundation for analysis in the health sciences*. Wiley

* Davis, A., and Appel, T.(1979). Bloodletting Instruments in the National Museum of History and Technology. *Smithsonian Institution Press*, Washington, D.C.(41): 34-36.

* De Lima, R.; Seabra, A.B., and Durán, N.(2012). Silver Nanoparticles: A Brief Review of Cytotoxicity and Genotoxicity of Chemically and Biogenically Synthesized Nanoparticles: Genotoxicity of Silver Nanoparticles. *Journal of applied toxicology*. 32(11):867-79.

* Dhanjal, S., and Cameotra, S. S. (2010). Aerobic biogenesis of selenium nanospheres by Bacillus cereus isolated from coalmine soil. *Microbial Cell Factories*. 9(1): 1-11.

* Diggle, S., and Whiteley, M. (2020). Microbe Profile: *Pseudomonas aeruginosa*:opportunistic pathogen and lab rat. *Microbiology*. 166 (1): 30–33.

* Długosz, O.; Sochocka, M.; Ochnik, M., and Banach, M.(2021). Metal and Bimetallic Nanoparticles: Flow Synthesis, Bioactivity and Toxicity. *Journal* of *Colloid* and *Interface Science*. 586(16): 807–818.

* Du, J.; Tang, J.; Xu, S.; Ge, J.; Dong, Y.; Li, H., and Jin, M.(2018). A Review on Silver Nanoparticles-Induced Ecotoxicity and the Underlying Toxicity Mechanisms. *Regulatory Toxicology* and *Pharmacology*. 98: 231–239.

* Dzrekoriska, J. R.;Bielecki, A. ,and Palinska, K. Z. (2009). Activity of selected hydrolytic enzymes from leeches (clitellata: Hirudinida) with different feeding strategies . *Biologia*. 64(2):370- 376.

* Electricwala, A.; Sawyer, R.T. ; Jones, C.P., and Atkinson, T.(1991). Isolation of thrombin inhibitor from the leech Hirudinaria manillensis. *Blood Coagulation* & *Fibrinolysi*. 2(1):83-9.

* Elliott, J.M. (2008). Population size, weight distribution and food in a persistent population of the rare medicinal leech, *Hirudo medicinalis*. *Freshwater Biology*. 53(8):1502–1512.

* Elliott J.M., and Kutschera U.(2011). Medicinal Leeches: Historical use, Ecology, Genetics and Conservation, *Freshwater Reviews*. 4(1), 21-41.

* Faedmaleki, F.; Salarian, A.A.; Ahmadi, A.H., and Rastegar, H. (2014). Toxicity Effect of Silver Nanoparticles on Mice Liver Primary Cell Culture and HepG2 Cell Line Iran. *Journal* of *Pharmacy Research*.13(1):235–242.

* Farooqui, M.D.A.; Chauhan, P.S.; Krishnamoorthy, P., and Shaik, J. (2021). Extraction of silver Nanoparticle from the leaf extracts of Clerodendrum inerme, *Digest Journal* of *Nanomaterials* and *Biostructures*. 5(1):43-49.

* Fehaid, A.; Fujii, R.; Sato, T., and Taniguchi, A.J .(2020). Silver nanoparticles affect the inflammatory response in a lung epithelial cell line. *Open Biotechnology Journal.*14(1): 113-123.

* Fernandez, J., Olea, N., Tellez, V., and Matte, C. (2010). Structure and development of the egg of the glossiphoniid leech Theromyzon rude: reorganization of the fertilized egg during completion of the first meiotic division. *Developmental Biology*. 137 (1): 142–154

* Firdhouse, M.J., and Lalitha, P. (2013). Biosynthesis of silver nanoparticles using the extract of alternanthera sessilis-antiproliferative effect against prostate cancer. *Cells Cancer Nanotechnol*. 4(6):137-143.

* Fischer, E. (1970). Lokalisation der Leucyl-aminopeptidase (LAP) beim Blutegel (*Hirudo medicinalis* L.) und beim Pferegel (*Haemopis sanguisuga* L.). *Acta Histochem*. 37(1): 170–175.

* Furtado, L.M.; Bundschuh, M., and Metcalfe, C.D.(2016). Monitoring the Fate and Transformation of Silver Nanoparticles in Natural Waters. Bull. *Environmental Contamination* and *Toxicology*. 97(4):449-55.

* Gagnon, C.; Turcotte, P.; Gagné, F., and Smyth, S.A.(2021). Occurrence and Size Distribution of Silver Nanoparticles in Wastewater Effluents from Various Treatment Processes in Canada. *Environmental Science* and *Pollution Research*. 28(46):1-8

* Gasic, G.J.; Iwakawa, A.; Gasic, T.B.; Viner, E.D., and Milas, L. (1984). Leech salivary gland extract from Haementeria officinalis, a potent inhibitor of cyclophosphamide- and radiation-induced artificial metastasis enhancement. *Cancer Research*. 44(12 Part 1):5670-6.

* Gasic, G.J. (1986). Method of treatment to inhibit metastasis. *Washington, DC: U.S. Patent* and *Trademark Office*. 4:588-587.

* Geraghty, R. J., Capes-Davis, A., Davis, J. M., Downward, J., Freshney, R. I., Knezevic, I., Lovell-Badge, R., Masters, J.R.W., Meredith, J., Stacey, G.N., Thraves, P., and Vias, M. (2014). Guidelines for the use of cell lines in biomedical research. *British Journal* of *Cancer*. 111(6): 1021-1046

* Ghanbari, H.; Viatge, H.; Kidane, A.G.; Burriesci, G.; Tavakoli, M.; Seifalian, A.M. (2009). Polymeric Heart Valves: New Materials, Emerging Hopes. Trends Biotechnol. 27(6): 359–367.

* Ghorbanpour, M.; Bhargava, P.; Varma, A., and Choudhary, D.K.(2020). Biogenic Nano-Particles and Their Use in Agro-Ecosystems; *Springer: Berlin/Heidelberg, Germany*. ISBN 978-981.

* Ghosh, S. (2019). Relevance of leech therapy in contemporary medicine: a mini-review. *Quest International Journal of Medical and Health Sciences*. 2(1): 8-12.

* Giovanni M.; Tay C.Y. and Setyawati M.I.(2014). Toxicity profiling of water contextual zinc oxide, silver, and titanium dioxide nanoparticles in human oral and gastrointestinal cell systems, *Environmental Toxicology*. 30(12):1459-69.

* Gottlieb, T., and Nimmo, G.R. (2011). Antibiotic resistance is an emerging threat to public health: an urgent call to action at the Antimicrobial Resistance Summit 2011. *Medical Journal* of *Australia*. 194(6):281-283.

* Goyak, K.M.O.; Laurenzana, E.M., and Omiecinski, C.J.(2010**).** Hepatocyte Differentiation. *Methods in Molecular Biology*. 640: 115–138.

* Graf, J.; Kikuchi, Y., and Rio, R.V.M. (2006). Leeches and their microbiota: naturally simple symbiosis models. *Trends in Microbiology*. 14(8): 365-371.

* Grafskaia, E.; Pavlova, E.; Babenko, V.V.; Latsis, I.; Malakhova, M.; Lavrenova, V.; Bashkirov, P.; Belousov, D.; Klinov, D.,and Lazarev, V.(2020). The Hirudo medicinalis microbiome is a source of new antimicrobial peptides. *International Journal* of *Molecular* Sciences. 21(19):7141.

* Green, P.A., and Shafritz, A.B. (2010). Medicinal leech use in microsurgery. Journal of *Hand Surgery*. 35(6):1019-21.

* Grunkemeier, G.L.; Jin, R., and Starr, A. (2006). Prosthetic Heart Valves: Objective Performance Criteria Versus Randomized Clinical Trial. Annals of *Thoracic Surgery*. 82(3): 776–780.

* Gurunathan, S.; Jeong, J.K.; Han, J.W.; Zhang, X.F.; Park, J.H., and Kim, J.H. (2015). Multidimensional effects of biologically synthesized silver nanoparticles in Helicobacter pylori, Helicobacter felis, and human lung (L132) and lung carcinoma A549 cells. *Nanoscale Research Letters*.10:35.

*Haleem, A.; Javaid, M.; Singh, R.P.; Rab, S., and Suman, R.(2023). Applications of nanotechnology in medical field: a brief review. *Global Health Journal*. 7(2): 70-77.

* Henderson, H.P.; Matti, B.; Laing, A.G.; Morelli, S., and Sully, L.(1983). Avulsion of the scalp treated by microvascular repair: the use of leeches for post-operative decongestion*British Journal* of *Plastic Surgery*. 36:235-239.

* Hildebrandt, J.P., and Lemke, S. (2011). Small bite, large impact–saliva and salivary molecules in the medicinal leech, Hirudo medicinalis. *Naturwissenschaften*, 98(12): 995-1008.

* Hodges, G. M., Linvingston, D. C., and Franks, L. M., J. (1973) . *Journal* of *Cell Science*. 12:887.

* Høiby, N.; Ciofu, O., and Bjarnsholt, T. (2010). *Pseudomonas aeruginosa* biofilms in cystic fibrosis". *Future Microbiology*. 5 (11): 1663–74.

* Hong, K.H.; Park, J.L.; Sul, I.H.; Youk, J.H., and Kang, T.J. (2006). Preparation of antimicrobial poly (vinyl alcohol) nanofibers containing silver nanoparticles. *Journal of Polymer Science*: *Polymer Physics*. 44(17):2468 - 2474.

* Huang, J.; Li, Q.; Sun, D.; Lu, Y.; Su, Y.; Yang, X.; Wang, H.; Wang, Y.; Shao, W.; He, N.; Hong, J., and Chen, C. (2007). Biosynthesis of Silver and Gold Nanoparticles by Novel SundriedCinnamomum Camphoraleaf. *Nanotechnology*. 18(10): 105104.

* Hyson, J.M. (2005). Leech therapy: a history. *Journal of the History of Dentistry*. 53(1): 25-27.

* Indergand, S., and Graf, J.(2000). Ingested blood contributes to the specificity of the symbiosis of Aeromonas veronii biovar sobria and Hirudo medicinalis, the medicinal leech. *Applied* and *Environmental Microbiology*. 66(11):4735-41.

* Iravani, S.; Korbekandi, H.; Mirmohammadi, S.V., and Zolfaghari, B.(2014). Synthesis of Silver Nanoparticles: Chemical, Physical and Biological Methods. *Research* in *Pharmaceutical Sciences*. 9(6): 385–406.

* Iravani, S.; Korbekandi, H.; Mirmohammadi, S.V., and Zolfaghari, B.(2014). Synthesis of Silver Nanoparticles: Chemical, Physical and Biological Methods. *Research* in *Pharmaceutical Sciences*. 9: 385–406.

* Ivask, A.; Elbadawy, A.; Kaweeteerawat, C.; Boren, D.; Fischer, H.; Ji, Z.; Chang, C.H.; Liu, R.; Tolaymat, T.; Telesca, D.; Zink J.; Cohen Y.; Holden P.A., and Godwin H.A. (2014). Toxicity mechanisms in Escherichia coli vary for silver nanoparticles and differ from ionic silver. *American Chemical Society Nano*. 8(1): 374–386.

* Jabir, M. S.; Nayef, U. M; Abdulkadhim, W. K., and Sulaiman G. M.(2019). Supermagnetic Fe3O4- PEG nanoparticles combined with NIR laser and alternating magnetic field as potent anti- cancer agent against human ovarian cancer cells. *Materials Research Express*. 6(11): 115412.

* Jain, D.; Daima, H.K.; Kuchhwaha, S., and Kothari, S.L. (2009).Plant mediated silver Nanoparticles using papaya fruit extract and evalution of their antimicrobial activites. *Digest journal of nanomaterials and biostructures.* 4(3):557-563.

* Jaganathan, J.; Kadarkarai, M.; Chellasamy, P.; Pari, M.; Devakumar, D.; Chithravel, V., and Balamurugan, C. (2016). Earthworm mediated synthesis of silver nanoparticles; A potent tool against hepatocellular carcinoma, Plasmodium falciparum parasites and malaria mosquitoes. *Parasitology International Journal*. 65(6): 276-284.

* Jemal, A; Bray, F.; Center, M.M.; Ferlay, J.; Ward, E., and Forman, D. (2011). Global Cancer Statistics. *Cancer Journal* for *Clinicians*. 61:69–90.

* Jeng, H.A., and Swanson, J.(2006). Toxicity of Metal Oxide Nanoparticles in Mammalian Cells. *Journal of Environmental Science* and *Health*, Part A Part A, 41(12): 2699–2711.

* Jennings, J.B., and van der Lande, V. (1967). Histochemical and bacteriological studies on digestion in nine species of leech. *Biology Bulletin*. 133(1): 166–183.

* Jha, K., Garg, A., Narang, R., and Das, S. (2015). Hirudotherapy in Medicine and Dentistry. *Journal of Clinical and Diagnostic Research* . 9 (12): 5-7.

* Jiang, H.S.; Yin, L.; Ren, N.N.; Xian, L.; Zhao, S.; Li, W., and Gontero, B. 2017). The Effect of Chronic Silver Nanoparticles on Aquatic System in Microcosms. *Environmental Pollution*. 223: 395–402.

* Johnson, M.A.A.; Shibila, T.; Amutha, S.; Menezes, I.R.A.; da Costa, J.G.M.; Sampaio, N.F.L., and Coutinho, H.D.M. (2020). Synthesis of Silver Nanoparticles

Using Odontosoria Chinensis (L.) J. Sm. and Evaluation of Their Biological Potentials. *Pharmaceuticals*. 13(4):66.

* Jorge de Souza, T.A.; Rosa Souza, L.R., and Franchi, L.P.(2019). Silver Nanoparticles: An Integrated View of Green Synthesis Methods, Transformation in the Environment, and Toxicity. *Ecotoxicology* and *Environmental*. 171: 691–700.

* Jyoti, K.; Baunthiyal, M., and Singh, A. (2016). Characterization of Silver Nanoparticles Synthesized Using Urtica Dioica Linn. Leaves and Their Synergistic Effects with Antibiotics. *Journal* of *Radiation Research* and *Applied Sciences*. 9: 217–227.

* Kadhem, H. A.; Ibraheem, S. A. ; Jabir, M. S.; Kadhim A. A.; Jihad,Z.J., and Florin, M.D.(2019). Zinc Oxide Nanoparticles Induces Apoptosis in Human Breast Cancer Cells via Caspase-8 and P53 Pathway. *Nano Biomedicine* and *Engineering*. 11(1): 35-43.

* Kalender, M.E.; Comez, G.; Sevinc, A.; Dirier, A., and Camci, C. (2010). Leech therapy for symptomatic relief of cancer pain. *pain medicines*. 11(3):443–5.

* Karataş, E.; Ceylan, M., and Dernekbaşı, S. (2022). Effects of mammalian blood with different glucose levels on reproduction, growth and survival of the southern medicinal leech, Hirudo verbana Carena, 1820. *Animal Reproduction Science*. 243:107030.

* Karthick, .; Namasivayam, S., and Abimanyu. (2011). Silver nanoparticle synthesis from Lecanicilliumlecanii and evolutionary treatment on cotton fabrics by measuring their improved antibacterial activity with antibiotics against *Staphylococcus aureus* (ATCC 29213) and *E. coli* (ATCC 25922) strains. *International Journal* of *Pharmacy* and *Pharmaceutical Sciences*. 3(4):190-195.

* Kaur, G., and Dufour, J.M.(2012**).** Cell Lines(Valuable tools or useless artifacts). *Spermatogenesis.* 2(1): 1–5.

* Kesharwani, P.; Gorain, B.; Low, S.Y.; Tan, S.A.; Ling, E.C.S.; Lim, Y.K.; C. M.; Lee, P. Y.; Lee, C. M.; Ooi ,C.H.; Choudhury, H., and Pandey, M**.** (2017). Nanotechnology based approaches for anti-diabetic drugs delivery. *Diabetes Research* and *Clinical Practice*. 136:52–77.

* Khan, I.; Saeed, K.; Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. Arab. J. Chem., 12(7): 908–931.

* Khan, M. S.; Alomari, A.; Tabrez, S.; Hassan, I.; Wahab, R.; Bhat, S.A.; Alafaleq, N.O.; Altwaijry, N.; Shaik, G.M.; Zaid, S.K.; Nouh, W.; Alokail, M.S., and Ismael, M.A. (2021). Anticancer potential of biogenic silver nanoparticles: a mechanistic study. *Pharmaceutics.* 13(5):707.

* Khanna, P., Kaur, A., and Goyal, D. (2019). Algae-based metallic nanoparticles: Synthesis, characterization and applications. *Journal of Microbiological Methods*. 163, 105656

* Kim, D.R., and Kang, K.W.(1998). Amino acid sequence of piguamerin, an antistasin-type protease inhibitor from the blood sucking leech Hirudo nipponia. *European journal of biochemistry*.254(3):692-7.

*Kim, E., and Viatour, P.(2020). Hepatocellular carcinoma: old friends and new tricks. *Experimental and Molecular Medicine*. 52(12) :1898–1907.

* Koeppen, D.; Aurich, M.; Pasalar, M., and Rampp, T.(2020). Medicinal leech therapy in venous congestion and various ulcer forms: perspectives of Western, Persian and Indian medicine. *Journal of Traditional* and *Complementary Medicine*. 10(2):104–9.

* Köhler, .CD., and Dobrindt, U. (2011). What defines extra intestinal pathogenic *Escherichia coli? International Journal of Medical Microbiology*.301:642-664.

* Kong, I.C.; Ko, K.-S., and Koh, D.-C.(2020). Evaluation of the Effects of Particle Sizes of Silver Nanoparticles on Various Biological Systems. *International Journal of Molecular Sciences.* 21(22): 8465.

* Kumar, P.; Singh, P.; Hussain, M., and Das, A.(2016). Synthesis of Silver Metal Nanoparticles Through Electric Arc Discharge Method: A Review. *Advanced Science Letters.* 22(1): 3–7.

* Kumar, V.; Yadav, S.C., and Yadav, S.K.(2010). Syzygium cumini leaf and seed extract mediated biosynthesis of silver nanoparticles and their characterization, *Journal* of *Chemical Technology and Biotechnology*. 8 (10):1301–1309.

* Kutschera, U., and Roth, M. (2006). Cocoon deposition and cluster formation in populations of the leech Hirudo verbana (Hirudinea: Hirudinidae). *Lauterbornia* 56: 5–8.

* Kutschera, U. (2012). The *Hirudo medicinalis* species complex. *The Science of Nature.* 99(5):433-4.

* Lai, S.; Zhang, Q., and Jin, L. (2023). Natural and Man-Made Cyclic Peptide-Based Antibiotics. *Antibiotics.* 12(1), 42.

* Lee, H.-Y.; Choi, Y.-J.; Jung, E.-J.; Yin, H.-Q.; Kwon, J.-T.; Kim, J.-E.; Im, H.-T.; Cho, M.-H.; Kim, J.-H.; Kim, H.-Y., and Lee B.-H. (2010). Genomics- Based Screening of Differentially Expressed Genes in the Brains of Mice Exposed to Silver Nanoparticles via Inhalation. *Journal* of *Nanoparticle Research*. 12(5): 1567–1578.

*Lee, S.H., and Jun, B.H. (2019). Silver Nanoparticles: Synthesis and Application for Nanomedicine. *International Journal of Molecula*. 20(4): 865.

* Lent, C.M.; Fliegner, K.H.; Freedman, E., and Dickinson M.H. (1988). Ingestive behavior and physiology of the medicinal leech. *Journal of Experimental Biology*. 137(1): 513-527.

* Lepeltier, E.; _Rijo, P.; Rizzolio, F.; Popovtzer, R.; _Petrikaite, V.; Assaraf, Y.G., and Passirani, C. (2020). Nanomedicine to target multidrug resistant tumors. *Drug Resist Updat*. 52: 100704.

* Li, Y., and Cummins, E.(2020). Hazard Characterization of Silver Nanoparticles for Human Exposure Routes. *Journal of Environmental Science and Health*, Part A. 55(6): 704–725.

* Lone, A.H.; Ahmad, T.; Anwar, M.; Habib, S.; Sofi, G., and Imam, H.(2011). Leech Therapy-A Holistic approach of treatment in Unani (Greeko-Arab) medicine. *Ancient Science of Life*. 31(1):31.

* Lu, Z.; Rong, K.; Li, J.; Yang, H., and Chen, R.(2013). Size-dependent antibacterial activities of silver nanoparticles against oral anaerobic pathogenic bacteria. *Journal of Materials Science*: *Materials in Medicine*. 24(6): 1465–1471.

* MacFaddin, J. E. (2000). Individual Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Lippincott Williams Wilkins, London.p:57-424.

* Magalhães, A.; Santos, L.; Lopes, L.; Estrela, C.; Estrela, C.; Torres, É.; Bakuzis, A.; Cardoso, P., and Carrião, M.(2012). Nanosilver Application in Dental Cements. *ISRN Nanotechnol*. 365438.

* Malik, B.; Astuti, D. A.; Arief, D. J. F., and Rahminiwati, M. (2019). A study on antioxidative and antimicrobial activities of saliva extract of Indonesian local leeches. In IOP Conference Series: *Earth and Environmental Science*. 251(1): 012061.

* Maltz, M.A., and Graf, J.(2011). The Type II Secretion System Is Essential for Erythrocyte Lysis and Gut Colonization by the Leech Digestive Tract Symbiont *Aeromonas veronii*. *Applied and Environmental Microbiology*. 77(2): 597–603.

* Maltz, M.A.; Bomar, L.; Lapierre, P.; Morrison, H.G.; McClure, E.A.; Sogin, M.L., and Graf, J. (2014). Metagenomic analysis of the medicinal leech gut microbiota. Front. *Microbiology*. 5, 151.

* Manjumeena, R.; Duraibabu, D.; Sudha, J., and Kalaichelvan, P.T. (2014). Biogenic Nanosilver Incorporated Reverse Osmosis Membrane for Antibacterial and Antifungal Activities against Selected Pathogenic Strains: An Enhanced Eco-Friendly Water Disinfection Approach. *Journal of Environmental Science* and *Health*, Part A. 49(10):1125–1133.

* Marambio-Jones, C., and Hoek, E.M.V.(2010). A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal* of *Nanoparticle Research*. 12, 1531–1551.

* Marchiol, L. (2012). Synthesis of Metal Nanoparticles in Living Plants. *Italian Journal of Agronomy*. 7(3): 37.

* Merzouk, A.; Ghawi, A.; Abdualkader, A.M.; Abdullahi, A.,and Alaama, M.(2012). Anticancer effects of medical Malaysian leech saliva extract (LSE). *Pharmaceutica Analytica Acta.* 15(1):2–6.

* Michalsen, A.; Roth, M.; Dobos, G., and Aurich, M. (2007). Medicinal Leech Therapy. *Stattgurt, Germany: Apple Wemding*;. Archives of facial plastic surgery: official publication for the American Academy of Facial Plastic and Reconstructive Surgery, Inc. and the International Federation of Facial Plastic Surgery Societies. 9(6):448-448.

* Mohammed, M.T.; jameel, A.A.; Abdullah, A.R., and Jumaah, H.M.(2022). Cytotoxic Effect of Silver Nanoparticles Produced from a Type of Pathogenic Bacteria Isolated from Clinical Samples in Iraq. *World Bulletin of Public Health* (WBPH). 11: 83-91.

* Monks, A.; Scudiero, D., Skehan, P.; Shoemaker,R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrich, M.; Campbell, H.; Mayo, J., and Boyd, M.(1991) Feasibility of high flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. *Journal of the National Cancer Institute.* 83(11): 757–766.

* Montinari, M.R., and Minelli, S.(2022). From ancient leech to direct thrombin inhibitors and beyond: New from old. *Biomedicine and Pharmacotherapy*.149: 112878.

* More, P.R.; Pandit, S.; Filippis, A.D.; Franci, G.; Mijakovic, I., and Galdiero, M. (2023). Silver Nanoparticles: Bactericidal and Mechanistic Approach against Drug Resistant Pathogens. *Microorganisms*. 11(369): 1-27.

* Moscato, S.; Ronca, F.; Campani, D., and Danti, S. (2015). "Poly(vinyl alcohol)/gelatin Hydrogels Cultured with HepG2 Cells as a 3D Model of Hepatocellular Carcinoma: A Morphological Study". *Journal of Functional Biomaterials*. 6 (1): 16–32.

* Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. ;65(1-2):55-63.

* Mumcuoglu, K.Y. (2014). Recommendations for the use of leeches in reconstructive plastic surgery. *Evidence- Based Complementary and Alternative Medicine*. 2014, Article ID 205929, 7 pages.

* Munshi, Y.; Ara, I.; Rafique, H., and Ahmad Z. (2008). Leeching in the history--a review. *Pakistan journal of biological sciences*. PJBS. 11(13): 1650–1653.

* Mutimer, K.L.; Banis, J.C., and Upton, J.(1987). Microsurgical reattachment of totally amputated ears. *Plast Reconstr Surg*. 79(4): 535-541.

* Murugesan, S.; Bhuvaneswari, S., and Sivamurugan, V. (2017). Green Synthesis, Chracterization of Silver Nanoparticles of a Marine Red Alga *Spyridia Fusiformis* and Their Antibacterial Activity. *International Journal of Pharmacy and Pharmaceutical Sciences*. 9(5): 192-197.

* Nadaroğlu, H.; Alayli Güngör, A., and İnce, S. (2017). Synthesis of Nanoparticles by Green Synthesis Method. *International Journal of Innovative Research and Reviews* (INJIRR). 1(1):6-9.

* Naghdi, M.; Taheran, M.; Brar, S.K.; Verma, M.; Surampalli, R.Y., and Valero, J.R.(2015). Green and Energy-Efficient Methods for the Production of Metallic Nanoparticles. *Beilstein Journal of Nanotechnology*. 6: 2354–2376.

* Nelson, M., and Graf, J. (2012) . Bacterial symbioses of the medicinal leech Hirudo verbena , *Gut Microbes*. 3(4): 322–331.

* Ndlovu, N.; Mayaya, T.; Muitire, C., and Munyengwa, N. (2020). Nanotechnology Applications in Crop Production and Food Systems. *International Journal of Plant Breeding and Crop Science*. 7(1): 603-613.

* Nikolic, M.; Sustersic, T., and Filipovic, N.(2018**).** In Vitro Models and On-Chip Systems: Biomaterial Interaction Studies With Tissues Generated Using Lung Epithelial and Liver Metabolic Cell Lines. Front. *Biotechnology* and *Bioengineering*. 6:120.

* Nwobodo, D.C.; Ugwu, M.C.; Anie, O.C.; Mushtak, T. S.; Al-Ouqaili, J.; Joseph, C.I.; Chigozie, U.V. and Saki, M. (2022). Antibiotic resistance: The challenges and some emerging strategies for tackling a global menace. J Clin Lab Anal. 36(e24655): 1-10.

* Nune, S.K.; Chanda, N.; Shukla, R.; Katti,K.; Kulkarni,R.R.; Thilakavathi,S.; Mekapothula,S.; Raghuraman Kannan, R., and Kattesh V., and Katti,K.V.(2009). Green nanotechnology from tea: phytochemicals in tea as building blocks for production of biocompatible gold nanoparticles, *Journal o*f *Materials Chemistry*. 19(19):2912–2920.

* Ojo, P. O.; Babayi, H.; Olayemi, I. K.; Peter, O. O.; Fadipe, L. A. Baba, E., and Izebe, K. (2018). Anti-Tubercular Activities and Molecular Characterization of Salivary Extract of Leech (Hirudo medicinalis) against Mycobacterium tuberculosis. *Journal of Tuberculosis Research.* 6 (1): 1-9.

* Omeje, M., and Kelechi, N. (2019). In Vitro Study on the Antimicrobial Activity of Curcuma longa Rhizome on Some Microorganism. *American Journal of Biomedical and Life Sciences*. 7(1): 1-5.

* Oves, M.; Aslam, M.; Rauf, M.A.; Kayyum, S.; Qari, H.A.; Khan, M.S.;Alam, M.Z.; Tabrez, S.; Pugazhendhi, A., and Ismail, I.M. (2018). Antimicrobial and anticancer activities of silver nanoparticles synthesized from the root hair extract of Phoenix dactylifera. *Materials Science and Engineering*. 89:429–443.

* Özkaya, D. (2023). Keratitis following leech therapy for periocular eczematous dermatitis: a case report. BMC Complement Med. *Therapies*. 23(1):124.

* Pablo, F.P., and Guy, S.S.(2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochemical Journal*. 384(Part 2):201-32.

* Pal, S.; Tak, Y.K., and Song, J.M. (2007). Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. *Applied and Environmental Microbiology*. 73(6):1712-20.

* Papavramidou, N., and Christopoulou-Aletra, H.(2009). Medicinal use of leeches in the texts of ancient Greek, Roman and early Byzantine writers. *Internal Medicine Journal.* 39(9):624-7.

* Patra, J. K., and Baek, K. H. (2014). Green nanobiotechnology: factors affecting synthesis and characterization techniques. *Journal of Nanomaterials*. 2014 : 1-12.

* Phillips, A.J.; Govedich, F.R., and Moser W.E. (2020). Leeches in the extreme: morphological, physiological, and behavioral adaptations to inhospitable habitats. International Journal for 130 Parasitology: *Parasites and Wildlife*. 12: 318-325.

* Płaza, G.A.; Chojniak, J., and Banat, I.M.(2014). Biosurfactant mediated biosynthesis of selected metallic nanoparticles. Int J Mol Sci. Płaza, G.A.; Chojniak, J., and Banat, I.M.(2014). Biosurfactant mediated biosynthesis of

selected metallic nanoparticles. *International Journal of Molecular Sciences*. 15(8): 13720-13737.

* Porshinsky, B.S.; Saha, S.; Grossman, M.D.; Beery Ii, P.R., and Stawicki, S.P.A. (2011). Clinical uses of the medicinal leech: a practical review. *Journal of Postgraduate Medicine*. 57(1): 65-71.

* Pourrahimi, M.; Abdi, M., Ghods, R. (2020). Complications of leech therapy. *Avicenna Journal of Phytomedicine*. 10(3): 222-243.

* Pratama, M.A.; Ramahdita, G., and Yuwono, A.H.(2019). The effect of silver nitrate addition on antibacterial properties of bone scaffold chitosan-hydroxyapatite. *AIP Conference Proceedings*. 2193(1): 020014- 6.

* Pugazhendhi, A.; Edison, T.N.J.I.; Karuppusamy, I., and Kathirvel, B.(2018). Inorganic nanoparticles: a potential cancer therapy for human welfare. *International Journal of Pharmaceutics*. 539(1–2):104–111.

* Qing, Y.; Cheng, L.; Li, R.; Liu, G.; Zhang, Y.; Tang, X.; Wang, J.; Liu, H., and Qin, Y.(2018). Potential antibacterial mechanism of silver nanoparticles and the optimization of orthopedic implants by advanced modification technologies. *International Journal* of *Nanomedicine*. 13: 3311–3327.

* Raineri, E. J. M.; Altulea, D., and van Dijl, J. M. (2022). *Staphylococcal* trafficking and infection-from ‘nose to gut’ and back. *FEMS Microbiology* Reviews. 46, fuab041

* Rafique, M.; Sadaf, I.; Rafique, M.S., and Tahir, M.B.(2017). A Review on Green Synthesis of Silver Nanoparticles and Their Applications. Artif. Cells *Nanomedicine, and Biotechnology*. 45(7):1272-1291.

* Rajendran, R.; Ganesan, N.; Balu, S.K.;. Srinivasan, A ; Premkumar, T.,and Devaki, T. (2015). Green synthesis, characterization, antimicrobial and cytotoxic effects of silver nanoparticles using Origanum heracleoticum leaf extract. *International Journal of Pharmacy and Pharmaceutical Sciences*. 7 (4) 288–293.

* Rajeshkumar, S.; Malarkodi, C.; Vanaja, M., and Annadurai, G. (2016). Anticancer and enhanced antimicrobial activity of biosynthesized silver nanoparticles against clinical pathogens. *Journal* of *Molecular Structure*.1116: 165–173.

* Ramirez, M.S., and Tolmasky, M.E. (2017). Amikacin: Uses, Resistance, and Prospects for Inhibition. *Molecules.* 22(2267):1-23.

* Rasigade, J.P., and Vandenesch, F.(2014). *Staphylococcus aureus*: a pathogen with still unresolved issues. *Infection*, *Genetics* and *Evolution*. 21:510-514.

* Rassadina, V.; Plazur, A.; Dandler, J., and Zoller, J.(2006). Growth of etiolated barley plants in weak static and 50 Hz electromagnetic fields tuned to calcium ion cyclotron resonance. *BioMagnetic* Research and *Technology*. 3:4:1.

* Reddy, C.A.(2007). Methods for general and molecular microbiology, 3rd Edition. *American Society for Microbiology*, Washington, D.C.

* Rezvani, E.; Rafferty, A.; McGuinness, C., and Kennedy, J.(2019). Adverse Effects of Nanosilver on Human Health and the Environment. *Acta Biomaterialia*., 94: 145–159.

* Rigbi, M.; Levy, H.; Iraqi, F.; Teitilbaum, M.; Orevig, M.; Alajoutijarvitz, A., and Alun, R. (1987). The saliva of the medicinal leech *Hirudo medicinalis*—1. Biochemical characterization of the high molecular weight fraction. Comparative Biochemistry and Physiology Part B: *Comparative Biochemistry*. 87(3): 567-573.

* Robbens, J.; Devriese, L.; Verstraete, K., and Heyndrickx, M. (2014). *Escherichia coli*. In P. Wexler (Ed.), *Encyclopedia of toxicology*. Elsevier Inc., Academic Press. 3rd ed. 2: 459-461

* Roe, D.; Karandikar, B.; Bonn-Savage, N.; Gibbins, B., and Roullet, J.-B.(2008). Antimicrobial Surface Functionalization of Plastic Catheters by Silver Nanoparticles. *Journal of Antimicrobial Chemotherapy*. 61(4):869–876.

* Roters, F.J., and Zebe, E. (1992b). Proteinase of the medicinal leech, Hirudo medicinalis: purification and partial characterization of three enzymes from the digestive tract. Journal of *Comparative Biochemistry* & *Physiology*. B 102(3): 627–634.

* Roters, F.J., and Zebe, E. (1992). Protease inhibitors in the alimentary tract of the medicinal leech *Hirudo medicinalis*: In vivo and *in vitro* studies. *Journal of Comparative Physiology* B. 162(1): 85-92.

* Saeb, A.T.M.; Alshammari, A.S.; Al-Brahim, H., and Al-Rubeaan, K.A.(2014). Production of silver nanoparticles with strong and stable antimicrobial activity against highly pathogenic and multidrug resistant bacteria. *Scientific World Journal.* 2014:1-9.

* Samberg, M.E.; Oldenburg, S.J., and Monteiro-Riviere, N.A. (2010). Evaluation of Silver Nanoparticle Toxicity in Skin *in Vivo* and Ker- atinocytes *in Vitro*. *Environmental Health Perspectives*. 118(3): 407 413.

* Samuel, M.S.; Jose, S.; Selvarajan, E.; Mathimani, T.,and Pugazhendhi, A.(2020). Biosynthesized silver nanoparticles using Bacillus amyloliquefaciens application for cytotoxicity effect on A549 cell line and photocatalytic degradation of p-nitrophenol. *Journal* of *Photochemistry and Photobiology*. 202:111642.

* Sanchez, A.G.; Victoria, M..B.; Portillo, A.L., and Sanchez, S.S.(2022). Antibacterial Activity in a Collection of amps from *Hirudo Medicinalis*. Journal of *Microbiology Reports*. 5(4): 35-38.

* Saratale, G.D.; Saratale, R.G.; Benelli, G.; Kumar, G.; Pugazhendhi, A.; Kim, D.-S., and Shin, H.-S. (2017). Anti-Diabetic Potential of Silver Nanoparticles Synthesized with Argyreia Nervosa Leaf Extract High Synergistic Antibacterial Activity with Standard Antibiotics Against Foodborne Bacteria. *Journal* of *Cluster Science*. 28(19): 1709–1727.

* Saravana, M.; Barik, S. K.; MubarakAli, D.; Prakash, P., and Pugazhendhi, A. (2018). Synthesis of silver nanoparticles from *Bacillus brevis* (NCIM 2533) and their antibacterial activity against pathogenic bacteria. *Microbial Pathogenesis* 116: 221-226.

* Sawyer, R.T.(1986). Leech Biology and Behaviour. Michigan: Clarendon Press;. Feeding, biology, ecology and systematics. Vol. 2.

* Schikorski, D.; Cuvillier-Hot, V.; Leippe, M.; Boidin-Wichlacz, C.;Slomianny, C.; Macagno, E.; Salzet, M., and Tasiemski, A. (2008). Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. *Journal of Immunology*. 181(2):1083–95.

* Sengottaiyan, A.; Aravinthan, A.; Sudhakar, C.; Selvam, K.; Srinivasan, P.; Govarthanan, M.; Manoharan, K., and Selvankumar, T. (2016). Synthesis and Characterization of Solanum Nigrum-Mediated Silver Nanoparticles and Its Protective Effect on Alloxan-Induced Diabetic Rats. *Journal* of *Nanostructure in Chemistry*. 6: 41–48.

* Seong, M., and Lee, D.G.(2017). Silver Nanoparticles against Salmonella enterica Serotype Typhimurium: Role of Inner Membrane Dysfunction. *Current Microbiology*. 74(6): 661–670.

* Settivari, R.S.; Rowlands, J.C.; Wilson, D.M.; Arnold, S.M., and Spencer, P.J.(2017).Chapter 32—Application of Evolving Computational and Biological Platforms for Chemical Safety Assessment. *In A Comprehensive Guide to Toxicology in Nonclinical Drug Development*, 2nd ed.; Faqi, A.S., Ed.; Academic Press: Boston, MA, USA,; pp. 843–873. ISBN 978-0-12-803620-4.

* Setyawati M.I.; Tay C.Y., and Leong D.T.(2013). Effect of zinc oxide nanomaterials-induced oxidative stress on the p53 pathway. *Biomaterials*. 34(38): 10133–10142.

* Shah, M.; Fawcett, D.; Sharma, S.; Tripathy, S., and Poinern, G. (. 2015). Green Synthesis of Metallic Nanoparticles via Biological Entities. *Materials*. 8: 7278–7308.

* Shahin, A.; Yüksel,N.C.; 2 Öncel, E.K.; Aksay, A.K.; Yılmaz, N., and Çiftdoğan1, D.Y. (2022). Clinical and Laboratory Features of Invasive Group A Streptococcal Infections: 8 Years' Experience. *Turk Arch Pediatr*. 57(1): 75–80.

* Shakouri, A., and Wollina, U. (2021). Time to change theory; medical leech from a molecular medicine perspective leech salivary proteins playing a potential role in medicine. *Advanced pharmaceutical bulletin*. 11(2): 261-266.

* Shanmuganathan, R.; Karuppusamy, I.; Saravanan, M.; Muthkumar, H.; Punnochamy, K.;Ramkumar, V., and Pugazhindhi A.(2019). Synthesis of Silver

nanoparticles and their biomedical applications - A comprehensive review. *Current Pharmaceutical Design*. 25(24):2650 2660.

* Sharma, V.K.; Yngard, R.A., and Lin, Y.(2009). Silver Nanoparticles: Green Synthesis and Their Antimicrobial Activities. Advances in *Colloid* and *Interface Science*. 145(1-2):83-96.

* Shikha, S.; Chaudhuri, S. R., and Bhattacharyya, M. S. (2020). Facile one pot greener synthesis of sophorolipid capped gold nanoparticles and its antimicrobial activity having special efficacy against Gram negative *Vibrio cholera*. *Scientific Reports.* 10(1), 1-13.

* Siddall, M.E.; Min, G.S.; Fontanella, F.M.; Phillips, A.J., and Watson, S.C.(2011). Bacterial symbiont and salivary peptide evolution in the context of leech phylogeny. *Parasitology*, 138(13): 1815–1827.

* Sig, A.K.; Guney, M.; Guclu, A.U., and Ozmen, E.(2017). Medicinal leech therapy—an overall perspective. I*ntegrative Medicine Research*. 6(4):337–43.

* Singh, P.; Kim, Y. J., and Zhang, D., and Yang, D.C.(2016). Biological Synthesis of Nanoparticles from Plants and Microorganisms. Trends Biotechnol., *International Journal of Molecular Sciences*. 34(7): 34, 588–599.

* Singh, R.., and Singh, D. (2014). Chitin Membranes Containing Silver Nanoparticles for Wound Dressing Application. *International Wound Journal*. 11(3): 264–268.

* Singh, N., Saha, P., Rajkumar, K., and Abraham, J. (2014). Biosynthesis of silver and selenium nanoparticles by Bacillus sp. JAPSK2 and evaluation of antimicrobial activity. *Der Pharmacia Lettre*. 6(6), 175-181.

* Singh, R.R., and Shukla, G. D.(2022). A brief Review on The Leech Cultivation and Applications of Leech Therapy in Pain Managem. *World Journal of Pharmaceutical Research*. 11(6): 540-546.

* Sivakumar, P.; NethraDevi, C., and Renganathan, S. (2012). Synthesis of silver nanoparticles using Lantana camara fruit extract and its effect on pathogens. *Asian Journal* of *Pharmaceutical* and *Clinical Research.* 5(3): 97-101.

*Solgi, R.; Raz, A.A.; Zakeri, S.; kareshk, A.T.; Yousef A., Jarehan A., and Djadid,N.A. (2021). Morphological and molecular description of parasitic leeches (Annelida: Hirudinea) isolated from rice field of Bandar Anzali, North of Iran. *Journal of Zoological Systematics and Evolutionary Research*. 23(1):101162.

* Solijonov, K., and Umarov, F. U. 2022. Ecology of leeches and gastropods of the lower akbuura river, Fergana valley, Uzbekistan. *Bulletin of the Iraq Natural History Museum*. 17(2):229-250.

* Song, J.Y., and Kim, B.S.(2009). Rapid biological synthesis of silver nanoparticles using plant leaf extracts , *Bioprocess* and *Biosystems Engineering*. 32(1): 79-84.

* Srikar, S.K.; Giri, D.D.; Pal, D.B.; Mishra, P.K., and Upadhyay, S.N.(2016). Green Synthesis of Silver Nanoparticles: *A Review. Genomic Sequence Classification*. 6(1): 34–56.

* Srivastava, A., and Sharma, R.(2010). A brief review on applications of leech therapy. *Archives of Applied Science Research*. 2:271–4.

* Steinmetz, L.; Geers, C.; Balog, S.; Bonmarin, M.; Rodriguez-Lorenzo, L.; Taladriz-Blanco, P.; Rothen-Rutishauser, B., and Petri-Fink, A. (2020). A

comparative study of silver nanoparticle dissolution under physiological conditions. *Nanoscale Advances*. 2: 5760–5768.

* Stetefeld, J.; McKenna, S. A., and Patel, T.R.(2016). Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophysical review. Springer*.8(4): 409- 427.

* Suffness, M., and Pezzuto, J.M.(1990). Assays related to cancer drug discovery, in: K. Hostettmann (Ed.), Methods in Plant Biochemistry: Assays for Bioactivity, *Academic Press, London*. 6: 71–133.

* Supino, R. (1995). MTT Assays. In: O'Hare, S., Atterwill, C.K. (eds*) In Vitro* Toxicity Testing Protocols. *Methods in Molecular Biology*. 43: 137- 149.

* Supraja, N.; Prasad, T.N.V.K.V.; Soundariya, M., and Babujanarthanam, R.(2016). Synthesis, characterization and dose dependent antimicrobial and anti-cancerous activity of phycogenic silver nanoparticles against human hepatic carcinoma (HepG2) cell line. *AIMS Bioengineering*. 3(4): 425-440.

* Supraja, N.; Prasad, T.N.V.K.V; Giridhara Krishna, T., and David, E.(2015). Synthesis, characterization, and evaluation of the antimicrobial efficacy of Boswellia ovalifoliolata stem bark-extract-mediated zinc oxide nanoparticles. *Applied Nanoscience.* 6: 581–590.

* Swidwinska-Gajewska, A.M., and Czerczak, S.(2014). Nanosilver—Harmful effects of biological activity. *Medical Prestigious*. 65(6): 831–845.

* Tanimizu, N., and Mitaka, T.(2016**).** Morphogenesis of Liver Epithelial Cells. *Hepatology Research*. 46(10): 964–976.

* Tasiemski, A.(2008). Antimicrobial peptides in annelids. *Invertebrate Survival Journal* .;5(1):75–82.

* Tasiemski, A., and Salzet, M. U.S. (2012). Use of extract of leeches as antibacterial agent. Washington, DC: U.S. Patent and Trademark Office, Patent No. 20,120,251,625.

* Tejamaya, M.; Römer, I.; Merrifield, R.C., and Lead, J.R.(2012). Stability of Citrate, PVP, and PEG Coated Silver Nanoparticles in Ecotoxicology Media. *Environmental Science* & *Technology*. 46(13):7011–7017.

* Tilahun, T.; Babu, H., and Berhane, M. (2020). Leech in the rectum causing lower GI bleeding in a four years old child: a case report. *Ethiopian Journal of Health Sciences*. 30(6): 1055-1057.

* Trefts, E.; Gannon, M., and David H. Wasserman, D.H.(2017).The liver. *Current Biology*. 27: R1147-R1151

* Tuszynski, G.P.; Gasic, T.B., and Gasic, G.J. (1987). Isolation and characterization of antistasin. An inhibitor of metastasis and coagulation. *Journal of Biological Chemistry*. 262(20):9718–23.

* Uddin, T.M.; Chakraborty, A.J.;,Ameer Khusro, A.; Zidan, B.R.M.; Mitra, S.; Talha Bin Emran, T.B.E., and Kuldeep Dhama, K. (2021). Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. *Journal of Infection and Public Health*. 14: 1750–1766.

* Ullah, A., Yin, X., Wang, F., Xu, B., Mirani, Z. A., Xu, B., Chan, M.W.H., Ali, A., Usman, M., Ali, N., and Naveed, M. (2021). Biosynthesis of selenium nanoparticles (via Bacillus subtilis bsn313), and their isolation, characterization, and bioactivities. *Molecules*. 26(18), 26185559 .

* Ünal, K. ; Erol, M.E., and Ayhan, H. (2023). Litreture Review on The Effectivness of medicinal Leech Therapy in The Wound Healing. *Ankara Medical Journal*. 2023;(1):151-164.

* van der Lande, V. (1972). Observations on histochemical "amino- peptidase" reaction in the intestine of certain species of leech (Annelida: Hirudinea), with particular reference to Erpobdella octoculata (L.). *Comparative Biochemistry* & *Physiology*. A 41: 813– 824

* Van Khanh, N., and Van Cu, P. (2019). Antibacterial activity of silver nanoparticles against aeromonas spp. and Vibrio spp. isolated from aquaculture water environment in Thua Thien Hue. Hue University *Journal of Science*: *Agriculture and Rural Development*. 128(3B), 5-16.

* van Meerloo, J.; Kaspers, G.J.L., and Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. In: Cree, I. (eds) Cancer Cell Culture. *Methods in Molecular Biology*. 731: 237- 245.

* Vasanth, S.B., and Kurian, G.A. (2017). Toxicity Evaluation of Silver Nanoparticles Synthesized by Chemical and Green Route in Different Experimental Models. *Artif. Cells, Nanomedicine, and Biotechnology*., 45(8): 1721–1727.

* Vismara, E.; Valerio, A.; Coletti, A.; Torri, G.; Bertini, S.; Eisele, G.; Gornati, R., and Bernardini, G.(2013). Non-Covalent Synthesis of Metal Oxide Nanoparticle–Heparin Hybrid Systems: A New Approach to Bioactive Nanoparticles. *International Journal of Molecular* Sciences. 14(7): 13463–13481.

* Wahab, R.; Siddiqui, M.A.; Saquib, Q.; Dwivedi, S.; Ahmad, J.; Musarrat, J.; Al-Khedhairy, A.A., and Shin, H.S.(2014). ZnO nanoparticles induced oxidative stress and apop-tosis in HepG2 and MCF-7 cancer cells and their antibacterial activity. *Colloids and Surfaces B*: *Biointerfaces.* 117: 267–276.

* Wallis, R.B.; Fidler, I.J.; Esumi, N., and Munich. (1992). Hirudin for the inhibition of cancer metastasis. Germany: European Patent Office, issued September 16;. *European Patent* No. EP 0503829.

* Wang, H.; Meng, F. M.; Jin, S. J.; Gao, J. W.; Tong, X. R.; and Liu, Z. C. (2022). A new species of medicinal leech in the genus Hirudo Linnaeus, 1758 (Hirudiniformes, Hirudinidae) from Tianjin City, China. ZooKeys. 1095: 83-96.

* Wenning, A. (1996). Managing high salt loads: from neuron to urine in the leech. *Physiological Zoology*. 69(4): 719-745.

* Whitaker, I. S.; Rao, J.; Izadi, D., and Butler, P. E. (2004). Historical Article: Hirudo medicinalis: ancient origins of, and trends in the use of medicinal leeches throughout history. *British Journal of Oral and Maxillofacial Surgery*. 42(2), 133-137.

* Wollina, U., Heinig, B., and Nowak, A. (2016). Medical leech therapy (Hirudotherapy), *Our Dermatology Online*. 7(1): 91-96.

* Wood, S.J.; Kuzel, T.M., and Shafikhani, S.H. (2022). Pseudomonas aeruginosa: Infections, Animal Modeling, and Therapeutics. *Cells*., 12(1):2-37.

* Xu, L.; Wang,Y.Y.; Huang, J.; Chen,C.Y.; Zhen-Xing Wang,Z.X., and Xie, H.(2020). Silver Nanoparticles: Synthesis, Medical Applications and Biosafety. *Theranostics*. 10(20): 8996–9031.

* Yadav, S., and Zhang, B. (2020). An uncommon cause of unilateral nasal bleeding. *Grande Medical Journal*. 2(1): 25-27.

* Yule, C.M.; and Yong, H.S.(2004). Freshwater Invertebrates of the Malaysian Region. Kaula Lampur: *Akademi Sains Malaysia*. ISBN: 983-41936-0-2.

* Zaidi, S.; Jameel, S.; Zaman, F.; Jilani, S.; Sultana, A., and Khan, S.A.(2011). A systematic overview of the medicinal importance of sanguivorous leeches. *Alternative Medicine Review*. 16(1):59-65.

* Zavalova, .LL.; Yudina, T.G.; Artamonova, I.I., and Baskova, I.P.(2006). Antibacterial non-glycosidase activity of invertebrate destabilase-lysozyme and of its helical amphipathic peptides. *Chemotherapy*. 52(3):158-60.

* Zavalova, L.L.; Baskova, I.P.; Lukyanov, S.A.; Sass, A.V.; Snezhkov, E.V.; Akopov, S.B.; Artamonova, I.I.; Archipova, V. S.; Nesmeyanov, V.A.; Kozlov, D. G.; Benevolensky, S. V.; Kiseleva, V. I.; APoverenny, A. M., and Sverdlov, E. D. (2000). Destabilase from the medicinal leech is a representative of a novel family of lysozymes. *Biochimica et Biophysica Acta*. 1478(1): 69-77.

* Zebe, E.; Roters, F.J., and Kaiping, B. (1986). Metabolic changes in the medical leech Hirudo medicinalis following feeding. C*omparative Biochemistry and Physiology*. A 84(1): 49–55.

* Zerbst-Boroffka, I., and Wenning, A. (1986). Mechanisms of regulatory salt and water excretion in the leech, *Hirudo medicinalis* L. *Zoologische Beiträge*, 30: 359-377.

* Zhang, Y.; Pan, X.; Liao, S.; Jiang. C.; Wang, L.; Tang, Y.; Wu, G.; Dai, G., and Chen, L. (2020). Quantitative Proteomics Reveals the Mechanism of Silver

Nanoparticles against Multidrug-Resistant Pseudomonas Aeruginosa Biofilms. *Journal* of *Proteome Research.*19:3109–3122.

* Zheng, M.; Wu, X.; Lu, C.; Zhangm, W.; Tang, S.; Luo, Y., and Liu, D. (2023). Polypept(o)ide-based bactericides: weapons against antibiotic-resistant bacterial infections. *Elsevier Materials today Chemistry*. 27 (101270).

Appendices

Zeta Potential Report

v2.3

Malvern

Sample Details

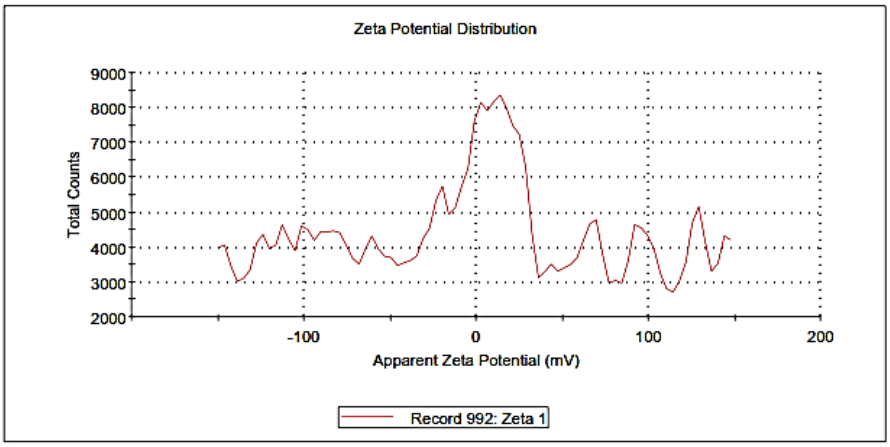
Sample Name:	Zeta 1		
SOP Name:	Water 25 DTS 1060.sop		
General Notes:			
File Name:	1401.dts	**Dispersant Name:**	Water
Record Number:	992	**Dispersant RI:**	1.330
Date and Time:	Saturday, January 26, 2002 11:52...	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

System

Temperature (°C):	25.0	**Zeta Runs:**	20
Count Rate (kcps):	72.3	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	9

Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-0.0607	**Peak 1:**	19.0	14.6	8.81
Zeta Deviation (mV):	138	**Peak 2:**	-3.54	11.0	7.28
Conductivity (mS/cm):	2.31	**Peak 3:**	-29.3	9.4	9.48
Result quality :	See result quality report				



Appendix(1): Zeta Nano Potential Pattern of Leech Salivary Extract Mediated Silver Nanoparticles.

Size Distribution Report by Volume

v2.2

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Sample Details

Sample Name: 40202-0096 1
SOP Name: Water 25 DTS 1060.sop
General Notes:

File Name:	1401.dts	**Dispersant Name:**	Water
Record Number:	991	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Saturday, January 26, 2002 ...

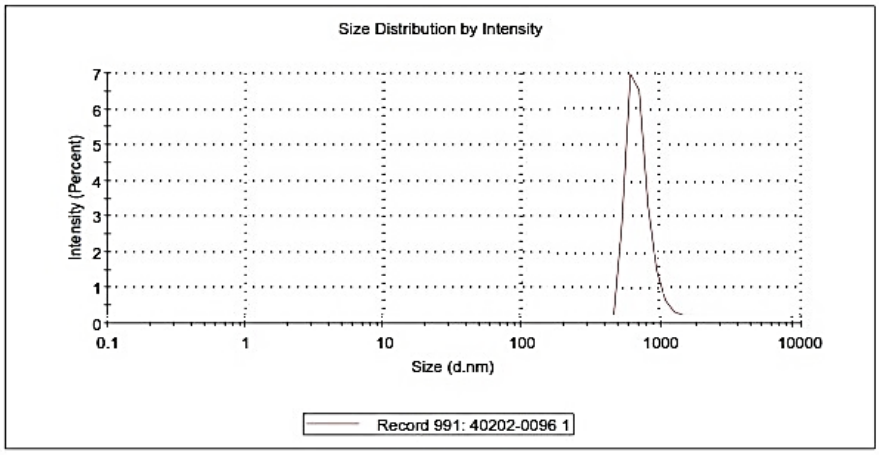
System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	200.2	**Measurement Position (mm):**	5.50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

Results

			Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm):	649.1	**Peak 1:**	649.1	100.0	0.4051
		Peak 2:	0.000	0.0	0.000
Intercept:	0.248	**Peak 3:**	0.000	0.0	0.000

Result quality : Refer to quality report



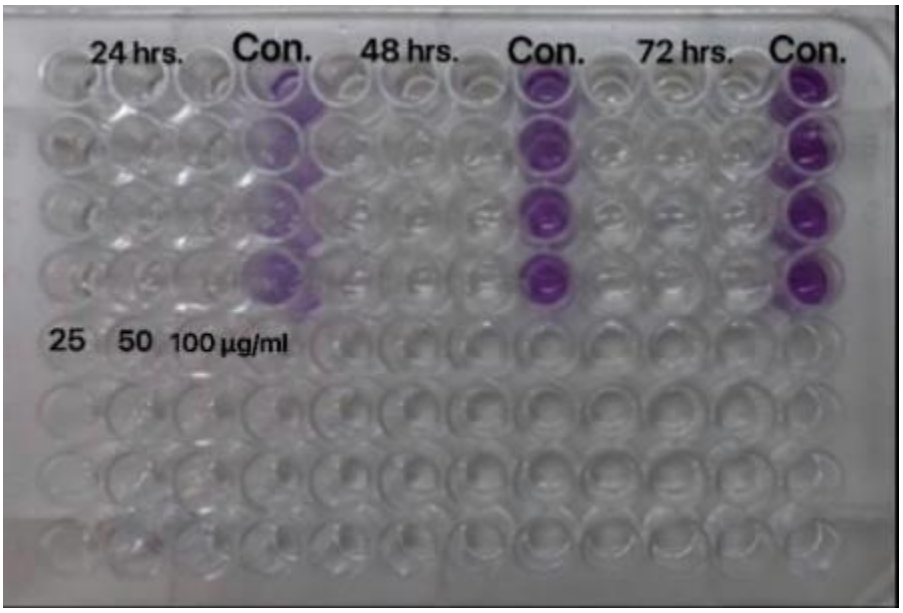
Malvern Instruments Ltd
www.malvern.com

Zetasizer Ver. 7.11
Serial Number : MAL1032666

File name: 1401
Record Number: 991
26 Jan 2002 11:56:40 PM

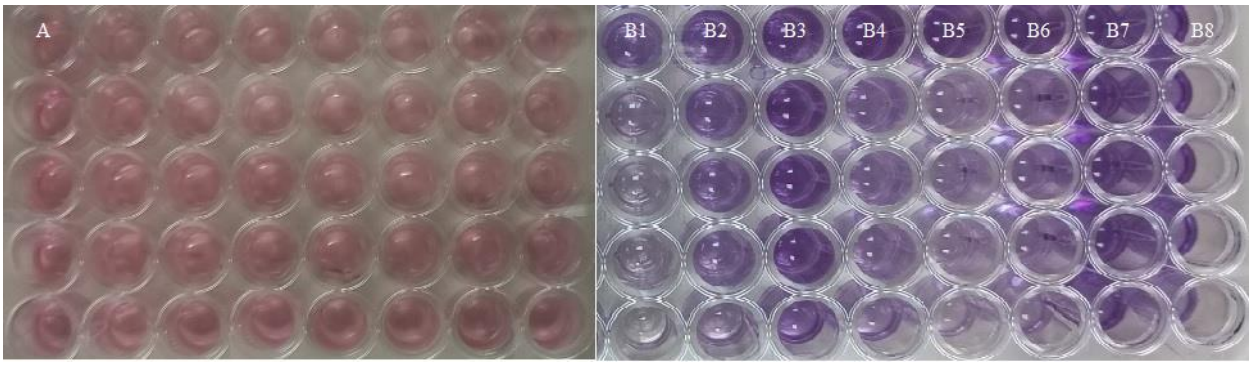
Appendix(2): Zeta Nano Size Distribution Pattern of Leech Salivary Extract Mediated Silver Nanoparticles.

MTT Assay



Con.: Control

25, 50, 100µg/ml: LSE-AgNPs Concentration

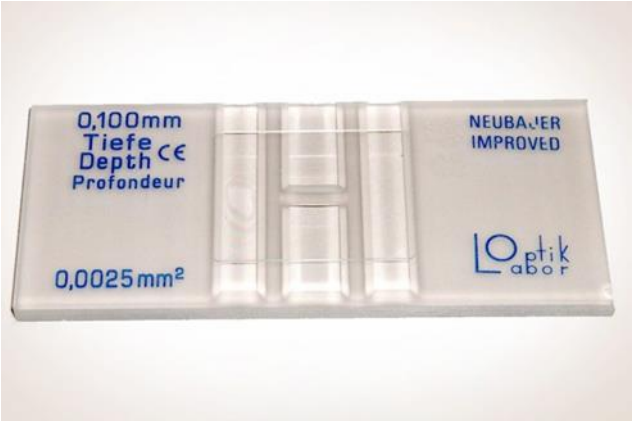


Appendix(3): MTT assay and formation of dissolved formazan. A) HepG2 cell coated in to the 96 well plate and contained with suitable culture medium (DMEM + 10% FBS), B) Formation of dissolved purple formazan using isopropanol.

Appendix(4):

Counting Chambers / Hemocytometers

We usually use from counting chambers which named hemocytometers.



The counting chamber has dimensions of 1 mm x 1 mm and are further subdivided into 0.05 mm x 0.05 mm squares.

1-Clean the hemocytometers with 70% ethanol and lens paper.

2- Coverslip is then gently placed atop the counting chamber.

3- Trypan blue should be added to the cell suspension (in a 1:1 ratio).

4- A small sample of cell suspension is taken using a pipette.

5-The cells are counted using a tally counter. Normally four 1 mm^2 squares are read and the results are averaged.

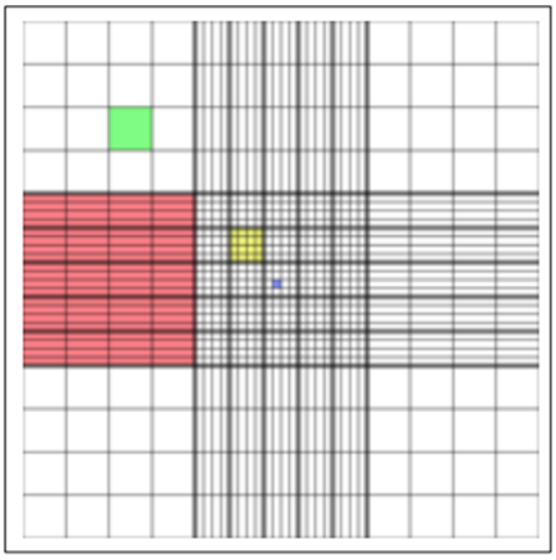


Fig 1. Schematic representation of a hemocytometer grid.

Red square = 1 mm², 100 nl
green square = 0.0625 mm², 6.25 nl
yellow square = 0.04 mm², 4 nl
blue square = 0.0025 mm², 0.25 nl at a depth of 0.1 mm

https://www.twinhelix.eu/radice/uploads/files/HowtoCountCellsAnOverviewofCellCountingMethods.pdf

الخلاصة

ان قدرة العلق الطبي على ازالة الفعالية المضادة للبكتيريا ادى الى جذب انتباه المعالجين التقليديين والأطباء والباحثين. يعد لعاب العلق الطبي والاحماض الدهنية للعلق من المكونات المهمة التي لها فعالية مضادة للبكتريا.

تحتل الطريقة البيولوجية لتخليق الجسيمات النانوية مساحة مهمة بسبب الفوائد الاقتصادية والصديقة للبيئة عند مقارنتها بطرق التخليق الفيزيائية والكيميائية. ومن هنا تهدف هذه الدراسة الى تقييم النشاط المضاد للبكتريا لمستخلص لعاب العلق الخام ومستخلص زيت العلقة وجسيمات الفضة النانوية لمستخلص اللعاب الخام ضد بعض البكتريا المسببة للأمراض وتحديد السمية الخلوية للجسيمات النانوية للعاب على خط الخلايا البشرية لسرطان الكبد.

تم جمع لعاب العلق بعد اثني عشر اسبوعا من التجويع وامتصاص المحلول البلعمي المتكون من المحلول الملحي بتركيز 0.15M والأرجانين بتركيز 0.001M من (80) علقة. كما تم جمع مستخلص الزيت الخام من (20) علقة باستخدام جهاز الكليفينجر المعتمدة على نظام التقطير. صنعت الجسيمات النانوية من لعاب العلق الطبي بيولوجيا وتم توصيفها بطريقة تشتت الضوء الديناميكي والمجهر الالكتروني الماسح. تم فحص النشاط المضاد البكتيري للعاب العلق الطبي الخام والجسيمات النانوية للعاب ومستخلص زيت العلق الخام ضد الانواع البكتيرية الامريكية, *Pseudomonas aeruginosa Escherichia coli* *Staphylococcus aureus and Streptococcus pyogenes* باستخدام طريقة نشر حفر الاكار والتركيز المثبط الادنى(MIC) والتركيز القاتل الادنى للبكتريا(MBC) . تم اختبار التأثيرات السامة للخلايا في المختبر للجسيمات النانوية المحضرة ضد خط الخلايا البشرية لسرطان الكبد وتم تأكيد النسبة المئوية لتثبيط الخلايا من خلال اختبار مجموعة ادوات الاختبار المعيارية(MTT).

كان تأثير مستخلص لعاب العلق الخام ومستخلص زيت العلق الخام غير فعال ضد العزلات البكتيرية المختبرة عند ($P \geq 0.05$) مقارنة بالمضادات الحياتية القياسية مثل (Amikacin) المستخدم كعنصر تحكم ايجابي تمنع نمو العزلات الاختبارية بحجم منطقة التثبيط 25.00±4.100mm لل *S.aureus* ، 23.00±4.400mm لل *E.coli* ،والمضاد الحياتي (Ampicillin) كعنصر تحكم ايجابي ضد*P. auroginosa* بقطر تثبيط 22.00±4.700mm و 25±5.800mm لل *S.pyogenes* على التوالي.

يرتبط تخليق جسيمات الفضة النانوية من لعاب العلق الطبي بالتحول اللوني لمستخلص اللعاب في المحلول عديم اللون إلى البني الذي يستمر لمدة 48 ساعة. ان جسيمات الفضة النانوية المخلقة من لعاب العلق الطبي تعتبر عوامل تثبيت واختزال. كانت نتائج تجربة تشتت الضوء الديناميكي (DLS) لتحديد حجم الدقائق النانوية للعاب بمتوسط حجم 649.1 نانومتر وبصماتها الكامنة في زيتا -0.060. تم استخدام FE-SEM لالتقاط صورة من سطح العينات. تم التقاط الصور بقدرة تكبير (× 10000) 10 KX و 35 KX)كشفت نتائجنا أن العينات تحتوي على جزيئات مشتتة جيدًا لها أشكال مربعة تقريبا. كانت أحجام الجسيمات النانوية بحدود 20 إلى 720 نانومتر بمتوسط قيمته 600 نانومتر. تم إجراء الاختبار بمستوى ثقة 95%.

اما اختبار تثبيط دقائق الفضة النانوية للعاب العلق الطبي وبحجم (15 µl) لنمو العزلات البكتيرية قيد الدراسة وبتراكيز مختلفة تراوحت بين (100, 200, 300 and 400µg/ml) مقارنة مع المضادات الحياتية(AM and CRO) كسيطرة موجبة. كانت النتائج ايجابية لجميع التراكيز ولجميع العزلات البكتيرية. وفقاً لنتائج اختبار التثبيط السابق وجدنا ان التركيز المناسب لدقائق الفضة النانوية للعاب العلق الطبي هو(100µg/ml) ومن هذا التركيز حضرت التخافيف اللازمة لاختبار MIC .

ففي اختبار ال MIC حيث يثبط (10 µl) من دقائق الفضة النانوية للعاب العلق الطبي نمو الاختبارات المعزولة من التركيز المرتفع(100µg/ml) إلى التركيز المنخفض(3.13µg/ml) لدقائق الفضة النانوية مقارنة بالمضادات الحياتية والسيطرة السالبة (PBS) على التوالي ، اظهرت نتائج التثبيط انه لا يوجد فرق معنوي في التثبيط بين التركيز(100µg/ml) للمادة النانوية المحضرة والتركيز(50µg/ml) على التوالي ، في حين يوجد فرق معنوي في نتائج التثبيط بشكل عام باختلاف التراكيز للمادة النانوية لكل عزلة بكتيرية قيد الدراسة الحالية وباحتمالية $p\leq0.05$ مقارنة مع المضادات الحياتية والسيطرة السالبة.

تم تحديد اقل تركيز مثبط لنمو البكتريا (MIC) واقل تركيز قاتل للبكتريا (MBC) لـدقائق الفضة النانوية باستخدام طريقة تخفيف المرق الدقيق. كانت قياسات(*P. aeruginosa, E. coli, S. aureus* MIC) على التوالي (50 مايكروغرام/مل) بينما كانت (MIC) للبكتيريا *S. pyogenes*(25 مايكروغرام/مل).كانت (MBC) *P.aeruginosa, E. coli, S.aureus* على التوالي (100 مايكروغرام/مل) على التوالي بينما كانت (MBC) للبكتيريا *S. pyogenes* (50 مايكروغرام/مل).

تقل فعالية خط الخلايا البشرية لسرطان الكبد بعد التفاعل مع تراكيز مختلفة من دقائق الفضة النانوية ويزداد هذا التأثير تدريجيا بين (24 و48) ساعة من التعرض.

تم استزراع خط الخلايا السرطانية لكبد الانسان ($HepG_2$) ولوحظت تفاصيلها الشكلية عن طريق المجهر الضوئي بعد الحضانة لمدة 24,48,72, ساعة في وجود تراكيز مختلفة (100, 50, 25 مايكروغرام/مل) من دقائق الفضة النانوية. تظهر الصور التمثيلية لخطوط الخلايا تغيرات ملحوظة في الشكل عند تراكيز دقائق الفضة النانوية(25 و50 مايكروغرام/مل) وبعد 48 ساعة بلغت قيمة IC_{50}(0.0124) للجسيمات النانوية 50مايكروغرام/مل تأثرت اشكال الخلايا عند هذه التراكيز بشكل كبير.

تشير النتائج التي تم الحصول عليها في هذه الدراسة إلى أنه يمكن استخدام دقائق الفضة النانوية للعاب العلق الطبي في علاج الأمراض المعدية الناجمة عن *P. aeruginosa , S. pyogenes, E. coli, S. aureus*.

وفقًا لمقايسات MTT وMBC، اظهرت أن تراكيز دقائق الفضة النانوية للعاب العلق الطبي المثبطة لنمو السلالات البكتيرية القياسية قيد الدراسة سامة لخطوط خلايا $HepG_2$.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل- كلية العلوم
قسم علوم الحياة

الفعالية المضادة للبكتريا والسرطان لجزيئات الفضة النانوية المصنعة من لعاب ديدان العلق

اطروحة مقدمة الى

مجلس كلية العلوم / جامعة بابل

كجزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/علوم الحياة

من قبل

لمى جاسم حمود حسون وتوت
بكالوريوس علوم حياة/ جامعة بابل (٢٠٠٢)
ماجستير علوم حياة/ جامعة بابل (٢٠١١)

بإشراف

الاستاذ الدكتور/ وجدان رضا محمود تاج الدين

١٤٤٥هـ ٢٠٢٣م