Characterization, Free Radical Scavenging and Antimicrobial Properties of *Hibiscus sabdariffa* Silver Nanoparticle

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CERTIFICATION

This is to certify that the study, "Characterization, free radical scavenging and antimicrobial properties of *Hibiscus sabdariffa* silver nanoparticles was carried out by Okorondu, Monica Mmachukwu. O. with registration number 20194199668 in partial fulfillment of the requirement for the award of Doctor of Philosophy (Ph.D.) Degree in Medical Biochemistry, Federal University of Technology Owerri.

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DEDICATION

This research work is dedicated to the loving memories of my parents Chief Sir Jacob and Lady Anthonia Anyika and my brother, Mr. Anthony Anyika.

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ABSTRACT

Characterization, free radical scavenging and evaluation of antimicrobial properties of *Hibiscus sabdariffa* silver nanoparticle were carried out. Nanoparticles were synthesized using biological method and the synthesized nanoparticles were characterized for further confirmation using methods such as UV-Visible Spectroscopy, Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Energy Dispersive Spectroscopy (EDX) and X-ray diffraction (XRD). Phytochemical screening of the crude and nano extracts of *Hibiscus sabdariffa* were also carried out using Gas Chromatography Mass Spectroscopy (Gc/Ms) in order to ascertain the biocomponents in the two extracts. Antimicrobial properties of the crude and nano extracts were analyzed using Spectrophotometric assay while the *in-vitro* free radical scavenging properties of the crude and nano extracts were equally analyzed. The result of the UV-Visible spectroscopy of the nano extract showed maximum light absorption at a wavelength of 420 nm. The EDX revealed 79.52% Ag (silver) and 20.48% O (oxygen). The microscopic studies gave agglomerated spherical silver nanoparticle with average size of 65.9 nm. The XRD result showed that the silver nanoparticle exhibited a polycrystalline face centered cubic (FCC) structure with an average crystallite size of 65.9 nm. The GC/MS result of the crude extract showed a total of fifteen (15) bio-compounds with 4-amino-1,2 naphtoquinone (33.82%) being the highest and hexadecenoic acid, methyl ester (0.36%) being the least in abundance. The GC/MS result of the nano extract showed a total of fifteen (15) organic compounds with Cis-vaccenic acid (46.37%) being the highest and Carbomethoxy vinyl methylamine and 6-octadecanoic acid (0.93%) being the least. The result of the total antioxidant capacity showed a higher antioxidant capacity of the nano extract compared to the crude extract. The crude extract recorded an IC_{50} of 0.42 mg/ml for DPPH, 0.38 mg/ml for Nitric oxide, 0.33 mg/ml for hydroxyl radical and 0.67 mg/ml for superoxide radical. The nano extract recorded an IC_{50} of 6.6 mg/ml for 2,2-diphenyl-1-picrylhydrazyl (DPPH), 0.72 mg/ml for nitric oxide radical, 0.28 mg/ml for hydroxyl radical and 0.66 mg/ml for superoxide radical. The antimicrobial properties of the crude and nano extracts showed that the nano extract exhibited improved antimicrobial potency against *Escherichia coli (E.* coli) and *Salmonella typhi* compared to the crude extract. The nano extract exhibited enhanced total antioxidant capacity and hydroxyl radical scavenging activity but lower DPPH and nitric oxide radical scavenging activity with improved antimicrobial efficacy against *E.coli* and *Salmonella typhi* compared to the crude extract. Both nano and crude extract of *Hibiscus sabdariffa* could serve as potential antioxidant and antimicrobial agents.

Keywords: Antioxidant, Antimicrobials, Free radicals, GC-MS, *Hibiscus sabdariffa*, Silver Nanoparticles.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Free radicals are unstable molecules containing unpaired electrons. They are formed naturally in the body due to metabolic processes, exposure to UV radiation, smoking, pollution and stress. Free radicals can cause cellular damage, oxidative stress, aging and age-related diseases. They are implicated in various diseases including cancer, diabetes, and neurodegenerative disorders. On the other hand, Antioxidants are molecules that neutralizes free radicals, prevents damage, reduces inflammation caused by free radicals and prevents chronic diseases. Antimicrobials are agents that inhibit or kill microorganisms. They reduce the risk of infection and are used to treat bacterial, viral and fungal infections.

Nanoparticles has been used in medical applications in the area of targeted drug delivery, and diagnostic imaging. Nanomedicine which employs nano devices and nano materials in the detection and treatment of infections and diseases has been on the increase in recent times due to certain factors such as: Advances in nanotechnology arising from improved synthesis and characterization methods (Singh, Kumar, Kumar & Yadav 2019) development of novel nanomaterials such as nanoparticles and nanotubes (Novoselov et al., 2020), Increasing demand for personalized medicine such as targeted therapies for specific diseases like cancer and genetic disorders (Sawyers et al, 2020), need for more effective and less toxic treatments (Collins et al., 2019).

Many approaches for nanoparticle synthesis have been discovered and improved since the development of nanotechnology but to overcome nanoparticles with limited properties such as

generation of toxic and hazardous chemicals that could harm the environment and human health (Singh et al., 2018), generation of large amount of waste that contributes to environmental pollution (Wang, Li & Zhang, 2019) and high energy requirements that will contribute to greenhouse gas emission (Zhang, Chen & Wang, 2020), green synthesis is usually applied and recommended. Green synthesis makes use of biological sources such as plants and microorganisms to synthesize nanoparticle. *Hibiscus sabdariffa*, a plant with traditional medicinal uses which has shown promising results (Wang & Chu, 2018) has been employed to synthesize silver nanoparticle (AgNps).

The quest for novel antioxidants and antimicrobial agents has led researchers to explore the potential of plant-mediated silver nanoparticles (AgNPS) (Sing et al., 2018), however a comprehensive study comparing the free radical scavenging and antimicrobial properties of *Hibiscus sabdariffa* silver nanoparticle with the crude extract is lacking.

It has been the interest of clinicians to develop safe drugs with increased efficacy, high performance and unique properties that conventional medicine could not provide. Drugs that can reach the target sites with 100% bioavailability and increased therapeutic effects, hence the need to explore nanotechnology through nanomedicine to serve in diagnosis, drug delivery and treatment. Silver which is a metallic nanoparticle possesses some medicinal qualities as was described by previous studies which when combined with medicinal plant such as *Hibiscus sabdariffa* will bring a better therapeutic effect. The biocomponents in the medicinal plant will act as in situ reducing and capping agent (Anupam et al., 2019)

Silver nanoparticles have been reported to exhibit antimicrobial activity against various pathogens (Ramesh & Mahesh, 2019)., while *Hibiscus sabdariffa* extract has been shown to possess antioxidant and anti-inflammatory properties (Alarcon et al., 2017). The combination of

AgNPS with *Hibiscus sabdariffa* extract may enhance their individual properties leading to a potent antioxidant and antimicrobial agent.

This study aims to investigate and compare the free radical scavenging and antimicrobial properties of *Hibiscus sabdariffa* crude and silver nanoparticle extract using various in-vitro assays. The characterization of these synthesized nanoparticles was performed using various techniques such as UV-Vis spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy dispersive x-ray spectroscopy (EDX) and X-ray diffraction (XRD). Phytochemical analyses of the crude and nano extracts were performed using Gas chromatography-mass spectroscopy method (GC/MS). *In vitro* free radical scavenging and antimicrobial properties of the crude and nano extracts were also analyzed. The findings will provide insights into the potential applications of *Hibiscus Sabdariffa* silver nanoparticle (AgNP) in pharmaceutical and biomedical fields.

1.2 Problem Statement

The quest for novel antioxidants and antimicrobials has led researchers to explore the potentials of plant mediated silver nanoparticles. There is an increased demand for personalized medicine and increased need for more effective and less toxic drugs with increased efficacy, high performance and unique properties that conventional medicine could not provide. There are increasing need for drugs that can reach the target sites with 100% bioavailability and increased therapeutic effects, hence the need to explore nanotechnology through nanomedicine to serve in diagnosis, drug delivery and treatment. The combination of AgNPS with *Hibiscus sabdariffa* extract may enhance their individual properties leading to a potent antioxidant and antimicrobial agent.

1.3 Objectives of the study

Main Objective

Characterization, Free Radical Scavenging and Antimicrobial Properties of *Hibiscus sabdariffa* silver nanoparticle.

Specific Objectives

i. Biological synthesis of the nanoparticle and its characterization using various methods such as U.V Spectroscopy, Scanning Electron Microscopy (SEM), Transmission electron microscopy (TEM), Energy Dispersive spectrophotometry (EDX) and X-ray Diffraction (XRD).

ii. Phytochemical screening of the native plant and the synthesized nanoparticle using Gas chromatography/ Mass Spectroscopy.

iii. Analysis of free radical scavenging properties of the synthesized nanoparticle and the crude extract.

iv. Analysis of the total antioxidant capacity of the synthesized nanoparticle and the crude extract.

v. Antimicrobial study of the synthesized nanoparticle and the crude extract against bacterial isolates such as *Staphylococcus. aureus, Escherichia. coli* and *Salmonella. typhi*

1.4 Justification

This study can lead to the development of natural based products with antioxidant and antimicrobial properties that has improved bioavailability and increased therapeutic effect useful in pharmaceuticals, cosmetics and food industries, offering sustainable alternatives to synthetic materials

1.5 Scope of the study/Limitation

This work Characterization, free radical scavenging and antimicrobial properties of *Hibiscus sabdariffa* silver nanoparticle has various objectives such as Biological synthesis of the nanoparticle and its characterization using various methods such as U.V Spectroscopy, Scanning Electron Microscopy (SEM), Transmission electron microscopy (TEM), Energy Dispersive spectrophotometry (EDX) and X-ray Diffraction (XRD), Phytochemical screening of the native plant and the synthesized nanoparticle using Gas chromatography/ Mass Spectroscopy, Analysis of free radical scavenging properties of the synthesized nanoparticle and the crude extract, Analysis of the total antioxidant capacity of the synthesized nanoparticle and the crude extract, Antimicrobial study of the synthesized nanoparticle and the crude extract against bacterial isolates such as *Staphylococcus. aureus*, *Escherichia. coli* and *Salmonella. typhi* which were implemented. Various results obtained showed that nanoparticles were actually synthesized and the synthesized nanoparticles showed unique antioxidant profile and an improved antimicrobial properties against *E.coli* and *Salmonella.typhi* compared to the crude extract of *Hibiscus sabdariffa* flower suggesting that the nano extract could serve as a potential antioxidant and antimicrobial agent useful in pharmaceuticals, cosmetics and food industries offering sustainable alternatives to synthetic materials. However, the limitations of the study are quite enormous, requiring expertise in multiple fields such as nanotechnology, botany and pharmacology, cost effective and time consuming. It has a limited scope which focused on *Hibiscus sabdariffa* extract and nano extract limiting generality of other medicinal plants and synthesizing nanoparticle in large scale may be quite challenging.

CHAPTER TWO

LITERATURE REVIEW

2.1. Nanoparticles

Nanoparticle as the name implies signifies microscopic particle of matter that is measured on a nanoscale, usually less than 100 nanometers. It is quite undetectable by the human eye and can exhibit significantly different physical and chemical properties to their larger material counterparts. It can occur naturally as biological particles such as bacteria and fungi, can be created as the by-products of combustion reactions or can be produced with purpose through engineering to perform specialized functions (Omowunmi, 2013). The principal features of nanoparticles are their shape, size, surface characteristics and inner structure. They can be encountered as aerosols (solids or liquids in air), suspensions (solids in liquids), or they can be encountered as emulsions (liquids in liquids). Their properties may be modified in the presence of certain chemicals. The composition of a specific nanoparticle can be very complex depending on what interactions it has had with other chemicals or particles and on its lifespan. The chemical processes taking place on the surfaces of nanoparticles are very complicated and largely remain unknown. Nanoparticles have different ways of interacting with each other. They can remain free or group together depending on the attractive or repulsive interactive forces between them. These interactions remain difficult to characterize. Nanoparticles suspended in gas tends to stick to each other more readily than in liquids.

Nanoparticles are now widely used in various applications such as in medicine, Engineering, Agriculture and various areas of human endeavor. Nanoparticles have numerous roles to play in medicine and therapeutics such as being used in diagnosis, detection and treatment of diseases.

They can also be used in drug delivery, to deliver drugs to target organs for instance, developing nanoparticles to assist in the transportation of chemotherapy drugs directly to cancerous growths and delivering of drugs to areas of damaged arteries in order to fight cardiovascular diseases. Some nanoparticles have been approved for use in medicine and therapeutics. The current approach for the implementation of Nano technological systems in different diagnosis and therapy is known as Nano medicine (Abuzer et al, 2020).

2.2. Nanoparticles in medicine

Nanomedicine can be classified into two main categories namely: Nano devices and Nano materials. Nano devices are nanoparticles created for the purpose of interacting with cells and tissues in order to carry out specialized functions such as microarrays and respirocytes.

Nano materials can be classified into Nano structured and Nano crystalline materials. Nano structured materials are those materials whose structural elements are in the dimension of 1-100nm range. They can be categorized into polymer based, non-polymeric and lipid-based nanoparticles. Polymer based nanoparticles include dendrimers, nanoparticles, miscelles, nano gels, protein nanoparticles and drug conjugates. Polymer based nanoparticles whether synthetic or natural, provide an alternative way for therapeutic applications due to certain characteristics such as biocompatibility, non-immunogenicity, nontoxicity and biodegradability (Crucho and Barros, 2017). In order to decrease the immunogenicity and toxicity of synthetic polymers, their polyester forms are used such as polylactic acid (PLA). Also, natural polymer based nanoparticles such as chitosan, gelatin, albumin and alginate seem to overcome toxicity issues and provide significant improvement in the efficiency of therapeutic agents compared to conventional methods of drug delivery. Polymeric nanoparticles are considered as the matrix system, whereby the matrix is uniformly dispersed. They can be classified as nanocapsules or

nanospheres depending on their composition. In nanocapsules, a unique polymer membrane encloses therapeutic agents, whereas the therapeutic agents are directly dispersed throughout or within the polymer matrix in nanospheres (Letchford, Liggins, Watson & Burt, 2009). The surface of polymeric nanoparticles could easily be modified and functionalized with a specific recognition ligand which increases the specificity of therapeutic agents in targeted tissues.

Non polymeric nanoparticles include carbon nanotubes, nano diamonds, metallic nanoparticles, quantum dots and silica based nano structured particles. Lipid based nanoparticles are also divided into liposomes and solid lipid nanoparticles. Majority of the nanoparticles clinically approved for therapeutic uses are the polymer based and the lipid-based components. Also, the nano crystalline particles formed by the combination of therapeutic agents in crystalline form are also used in clinical applications.

2.2.1 Dendrimers

These are widely used polymers in clinical applications. They are used due to their hyper branched, compartmentalized structure. The number of branches in this polymer-based nanoparticle is controlled to allow their fabrication in very small sizes (1-5nm). Fabrication can be done by polymerization in spherical shape which leads to the formation of cavities within the dendrimer molecule. High entrapment efficiency is obtained with high generation dendrimers such as dendrimers containing more than sixty-four (64) surface groups when compared to smaller dendrimers, and they are used for the delivery of therapeutic agents. Additionally, dendrimers contain free end groups that can be easily modified and used for the conjugation of biocompatible compounds to enhance low cytotoxicity and high bio-permeability of the molecule. Such surface modifications can also be applied to improve the target specific delivery of therapeutic agents (Abuzer et al., 2020). Assembling dendrimers by either encapsulation or

complexation makes them attractive vehicles for the concomitant delivery of biologically active molecules such as vaccines, drugs and genes to the target locations and currently, mono or copolymers such as polyethyleneimine, polyamidoamine, poly(propyleneimine), chitin, etc. are used for therapeutic applications in the form of dendrimers (Hsu, Bugno, Lee & Hong, 2017), (Palmerston, Pan & Torchilin, 2017).

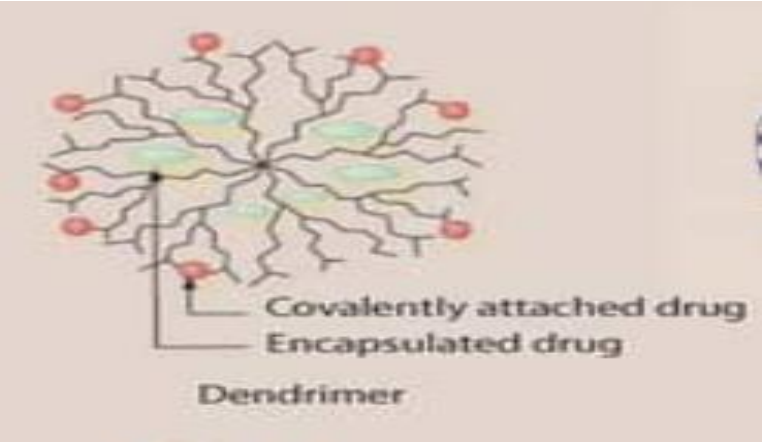

Figure. 2.1 Structure of a Dendrimer
Source: Bamrungsap et al., 2012

2.2.2 Micelles

Polymeric micelles are mostly used for the systemic delivery of water insoluble therapeutic agents. They are usually formed in solutions as aggregates. Their component molecules are arranged in spheroidal structure in which a mantle of hydrophilic groups surrounds hydrophobic cores. The existence of hydrophilic surface contributes to their protection from nonspecific uptake by the reticuloendothelial system ensuring their high stability within physiological systems. The hydrophobic core of polymeric micelles can physically trap the water insoluble, hydrophobic therapeutic agents. The component molecules can also be covalently linked to this hydrophobic core and the dynamic structure of polymeric micelles provides a prominent delivery

system for therapeutic agents, which allows versatile loading capacity, conjugation of targeted ligands and lower rate of dissolution (Ahmad, Shah, Saddiq & Kraatz., 2014).

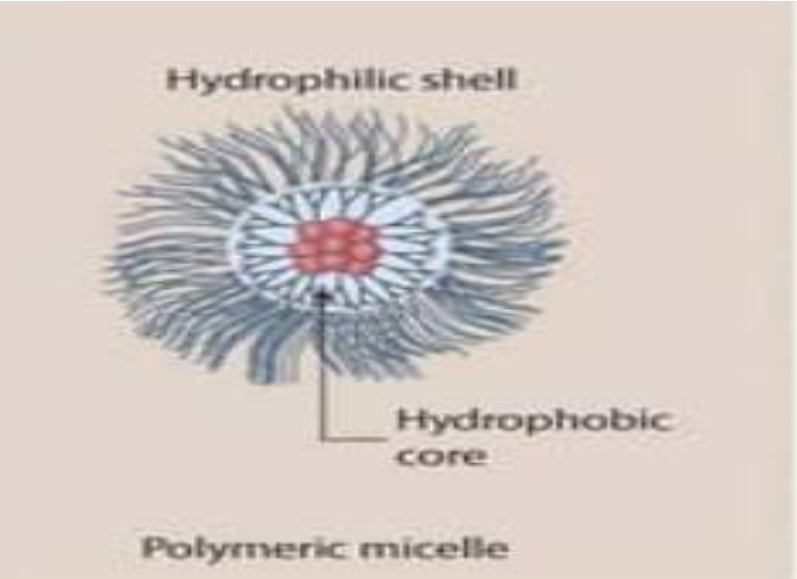

Figure 2.2 Polymeric micelle
Source: Bamrungsap et al, 2012

2.2.3 Drug Conjugates

Conjugation of polymers with drug molecules is generally used for low molecular weight agents particularly in cancer treatment. This conjugation increases the overall molecular weight of drugs which induces the pharmacokinetic disposition in the cells. Polymer drugs conjugates serve as carriers with high solubility and stability and promotes an enhanced permeability and retention effects (EPR) in cancer cells (Markovky, Baabur-Cohen & Satchi-Fainaro, 2014). Covalently conjugated polymer drugs are shown to be more reliable for sustained drug release and enhanced drug capacity (Yang, Mondal, Wen & Mahato., 2017). There are pH sensitive polymeric drug conjugates which are made by utilizing pH responsive chemical bonds between polymer and drug. Thus, the pH sensitivity of the nanoparticle is used for controlled drug release in the tumor

site due to its acidic environment (Pang et al., 2015). It was also reported that polymeric drug conjugates increase the bioavailability of the drug as shown with paclitaxel and doxorubicin combination therapy (Yang et al., 2017).

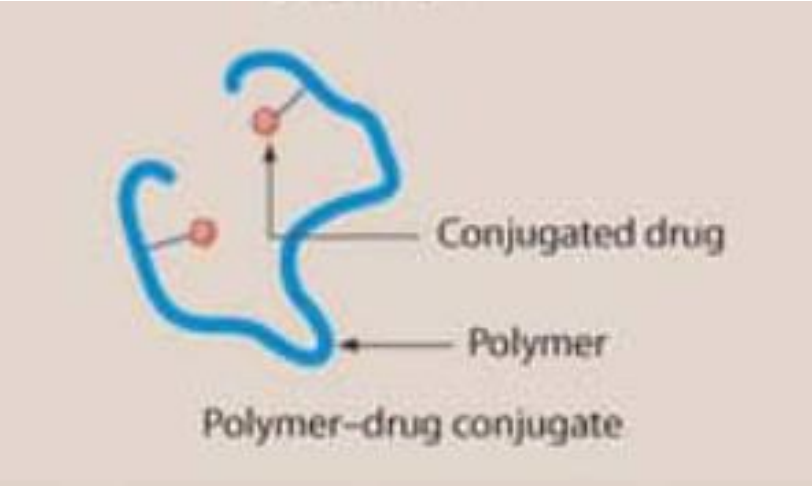

Figure 2.3 Polymer-drug conjugate
Source: Bamrungsap *et al*., 2012

2.2.4 Protein Nanoparticles

Viruses are natural carrier systems and very efficient for transferring their genetic material which is encapsulated by the capsid proteins. Virus-like particles (VLP), a type of protein nanoparticles, are defined as nano carrier systems, which have morphologically similar virus isolated structure, but do not include the viral genetic material (Tu et al., 2015). Also, caged proteins (CP) are defined as self-assembled protein nanostructures, which are morphologically similar to viruses. The VLPs and CPs are attractive nano carrier systems for the development of vaccines for cancer because they can induce antigen specific immune responses against cancer cells (Neek et al., 2019). There are protein nanoparticles made by self-assembly of protein polymers, which are isolated proteins from animal or plant origin such as collagen, gelatin, silk, albumin, elastin and soy. Through genetic engineering, protein polymers are self -assembled into functional drug delivery carriers with advantages of polymer-based nanoparticles (Taslimi et al.,2017). Abraxine,

which is an FDA (US food and drug Administration)-approved protein nanoparticle drug enables paclitaxel delivery by albumin. On the other hand, an HIV vaccine made from VLPs led to critical developments which accelerated research on protein nanoparticles for clinical use (Herrera, Champion & Zaritzky, 2015).

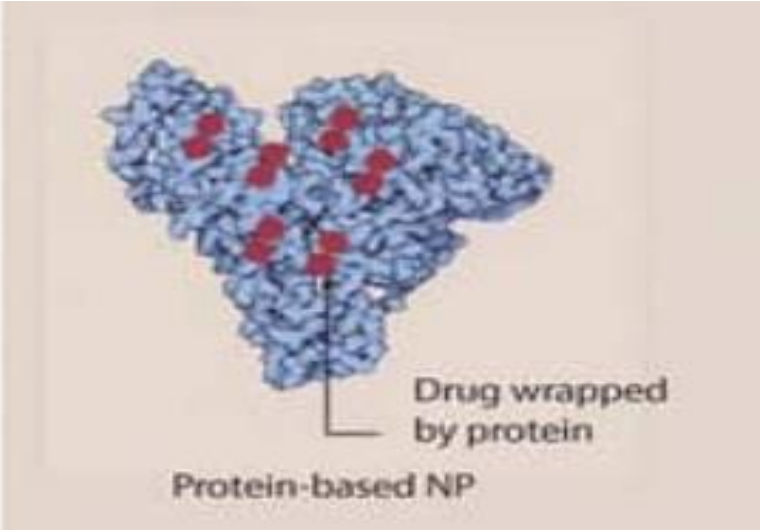

Figure 2.4 Protein-based Nanoparticle
Source: Bamrungsap et al., 2012

2.2.5 Nanogels

The gels are defined as non-fluid colloidal or polymeric networks that swell when in contact with fluid. A nanogel is considered as a particle of gel with similar properties. The swelling property with flexible size and high-water content of the nanogels is the result of physically or chemically cross-linked natural or synthetic polymers (Sharma, Yingard & Lin, 2016). The first reported nanogel was prepared by physical crosslinking of amphiphilic polysaccharides, where cholesterol-bearing pullulans (polysaccharide derived from starch) are self-assembled by hydrophobic interactions into nanogels in water (Tahara et al., 2015). The nanogels have some advantages compared to other nano-carrier systems such as decreased untimely drug leakage, encapsulating various therapeutic molecules in the same formulation and easy administration

through parental or mucosal routes. The nanogels are used in various applications, including biosensors, biochemical separation, cell culture, bio-catalysis, drug delivery, antitumor therapy and so on. Among all these, delivery of therapeutics such as nucleic acids, cytokines and nasal vaccines are the most widely studied applications of nanogels (Tahara et al., 2015).

2.3 Non-Polymeric Particles

2.3.1 Carbon Nanotubes

These are carbon-based tubular structures between 1-100 nm in length. These structures can be obtained by wrapping a single layer of graphite called graphene into a seamless cylinder. Their configuration includes single-walled nanotubes (SWNTs), multi-walled nanotubes (MWNTs) and C60 fullerenes. Their size and stable geometric shape makes them an attractive non-polymeric carrier for therapeutic agents especially, SWNTs and C60 fullerenes which have internal diameters of 1-2 nm, equivalent to about half of the average DNA helix diameter (Reilly et al., 2007). The SWNTs and MWNTs can enter cell by endocytosis or by direct insertion through the cell membrane. Fullerenes differ in the arrangement of their graphite cylinders and the presence of a high number of conjugated double bonds in their core structure. Experiments with fullerenes have shown that they can be used for the delivery of therapeutics like antibiotics, antiviral and anticancer agents (Mroz et al.,2007). They can also protect the injured mitochondria by providing free radicals (Cai et al., 2008). This feature, allows for the tissue-selective targeting of mitochondria that can be used for delivering therapeutic agents (Markovic et al, 2008).

2.3.2 Nano Diamonds (NDs)

Nano diamonds (NDs) are members of carbon-based nanomaterials with a diameter of less than 100 nm. They have different shapes with two types of discrete facets, which are generated from

various methods such as detonation, chemical vapour deposition (CVD), and high pressure/high temperature methods (Man et al., 2013). The oldest and most commonly used nano diamond preparation is the detonation method. In detonation method, nano diamonds are produced by activating a controlled explosion on carbon containing precursors in a closed chamber. NDs that are produced in this method usually contain sp^2 carbon on the surface and the electrostatic potential of surface is dependent on shape and structure of the NDs (Ho, Wang, & Chow, 2015). The CVD method is preferable for depositing NDs onto various substances as thin films. The produced ND thin films are of high quality with low defects (Chakrabati et al., 1998). NDs have unique properties, including surface electrostatic properties, low cytotoxicity, a chemically inert core, and low photo-bleaching by the addition of nitrogen defects and can be functionalized by immobilization of various types of biomolecules, which makes them remarkable for biomedical applications such as magnetic resonance imaging (MRI), synthesis of contact lenses and drug delivery for cancer therapy. NDs can be coupled with gadolinium (Gd) (111) as a contrast agent for MRI, and the signal generated from this complex is several times higher compared to Gd (111)-based contrast agents (Ho, Wang & Chow, 2015).

2.3.3 Metallic Nanoparticles

Metallic nanoparticles used in medical applications are 1-100 nm in size and mostly made up of cobalt, nickel, iron, gold and their respective oxides like magnetite, cobalt ferrite and chromium dioxide. They can be synthesized and modified with versatile functional chemical groups that allow them to be decorated with various molecules including therapeutic agents, biological molecules like peptides, proteins and DNA. As a carrier, they provide unique characteristics such as magnetic properties besides stability and biocompatibility. Magnetic nanoparticles can be targeted to a specific location in the body by using an external magnetic field. Magnetic

susceptibility, which is the ratio of induced magnetization to the applied field is an important parameter for their medical use. For instance, super-paramagnetic iron oxide nanoparticle (SPIONs) have a large magnetic susceptibility and thus they are widely used in clinics as contrast agents for magnetic imaging (Cuenca, Soga, Asencio & Perez, 2006). Also, super-paramagnetic properties facilitate the stable delivery of therapeutic agents to the body/cell and proper accumulation at the target tissue providing a reproducible and safe treatment approach (Reilly et al., 2007). When metallic nanoparticles are subjected to an alternating magnetic field, they can produce heat that is called magnetic hyperthermia, which enables their use in the ablation of tumors for cancer treatment (Andocs, Szasa, Asencio & Perez, 2009). Gold nanoparticles (AuNPs) are widely used metallic nanoparticles, especially in cancer diagnosis and therapy. The reason for this is the unique optical and localized surface plasmon resonance (LSPR) and relatively low cytotoxicity due to the inert nature of gold. When the light with appropriate wavelength is administered to AuNPs as external stimuli, due to the LSPR property, they exhibit photothermal conversion and heat up the targeted tumor tissue to kill cancer cells. AuNPs are used for drug delivery where the light irradiation can trigger the drug release, which is at the targeted site (Mura et al., 2013; Tian, Li & Wang, 2016). AuNPs optical and LSPR properties can be tailored for applications such as imaging, optical and electrochemical detection, diagnosis and photothermal therapy (Singh et al., 2018).

In as much as these noble metals have been used in nanotechnology especially nanomedicine, silver nanoparticles (AgNPs) are one of the most important and fascinating nanomaterial among the several other metallic nanoparticles. They are increasingly used in different fields such as for industrial purposes, food, health care, consumer and medical purposes due to their unique physical and chemical properties which includes- optical, thermal, high electrical conductivity and biological properties (Gurunathan, Park, Han & KIM, 2015). They have also been applied as

antibacterial agents, industrial, household, cosmetics and health-care products. Also, in food industry, diagnostics, medical device coatings, optical sensors, pharmaceutical industries, orthopedics, drug delivery vehicle and also as an anticancer agent. AgNp was recently used to enhance the tumor-killing effects of anticancer drugs (Chernousova & Epple, 2013). Nanosized metallic particles are quite unique and can considerably change their physical, chemical and biological properties due to their surface-to-volume ratio and therefore have been exploited for various purposes (Sharma, Yingard & Lin, 2009). In order to explore maximally their uses and satisfy the requirements of AgNps, various methods have been widely adopted for the synthesis. It was observed that the conventional physical and chemical methods seem to be very expensive and hazardous (Gurunathan et al., 2009). In contrast, biologically synthesized AgNPs show higher yield, solubility and stability than the other two methods hence seem to be simple, rapid, non-toxic, dependable and have green approaches that can produce well defined size and morphology. Therefore, biological synthesis of AgNPs shows much promise.

After the synthesis of nanoparticle or any nanomaterial for the purpose of nanomedicine, human welfare or in the health care industry, it is quite necessary to characterize it before application (Lin, Lin, Wang & Sridhar, 2014).The characteristic feature of nanomaterials such as shape, size, surface area, solubility, aggregation etc. need to be evaluated before assessing toxicity or biocompatibility (Mudrock et al., 2008). Many analytical techniques can be used to evaluate the synthesized nanomaterial such as ultraviolet visible spectroscopy (UV-vis spectroscopy), X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS), scanning electron microscopy (SEM), atomic force microscopy (AFM) etc.

The biological activity of AgNPs depends on factors such as surface chemistry, shape, size distribution, particle morphology, particle composition, agglomeration, dissolution rate and particle reactivity in solution. The type of reducing agent used for the synthesis of AgNPs is also another crucial factor used to determine cytotoxicity (Carlson et al., 2008).

All these physicochemical properties enhance the bioavailability of therapeutic agents after both systemic and local administration (Jo, Kim, Lee, & Kim, 2015); (Staquicini et al.,2011). Also, these physico chemical properties can affect cellular uptake, biological distribution, penetration into biological barriers and overall therapeutic effect (Duan & Li, 2013); (Albanese, Tang & Chan, 2012). In other words, the development of AgNPs with controlled structures that are uniform in size, morphology and functionality are essential for various biomedical applications (Sriram, Kanth, Kalishwaralal and Gurunathan, 2010).

2.3.4 Quantum dots

They are tiny particles or nanocrystals of semi conducting material with diameters in the range of 2-10 nm. Quantum dots produce distinctive fluorescence colours that are partly the result of unusually high surface-to-volume ratios for such particles. The core structure of quantum dots determine the color emitted, while the outer aqueous shell can be used for conjugation of biomolecules such as peptides, protein or DNA (Iga et al., 2007). Quantum dots can be used for tracking therapeutic agents within the cells/tissues due to their narrow emission, bright fluorescence and high photo-stability (Bailey, Smith & Nie, 2004)

2.3.5 Silica-Based Nanoparticle

Silica-based nanoparticles have considerable advantages in nanotechnology due to their application in designing complex systems and cost effectiveness. Their specific surface

characteristic, porosity and capacity for functionalization make them attractive tools for therapeutic delivery (Chen, Chen, Li & Dong, 2018). Silica nanoparticles have a large surface area that is covered with polar silanol groups that are favourable for water adsorption and stability improvement of therapeutic agents. In addition, silica-based nanoparticles have the ability to interact with nucleic acids, which allow their use as targeted delivery vehicles (Bharali et al., 2013). Their nanopore size and density can be improved to achieve a constant delivery rate. Also, encapsulation of therapeutic agents within silica-based nanoparticles provides solid media for the delivery of agents. Pores of silica nanoparticles can be capped with various stimuli-responsive molecules to increase the rate of drug release in the targeted tissue, for example, mesoporous silica. Nanoparticles scapped with β-cyclodextrin were developed to release the encapsulated drug at the acidic tumor tissue (Mura et al., 2013). Furthermore, these nanoparticles are used as additives in pharmaceutical production, to improve the mechanical properties and biocompatibility of the product.

2.4 Lipid-Based Nanoparticles

2.4.1 Liposomes

Liposomes are vesicles synthesized through the hydration of dry phospholipids. They can be prepared in distinct structure, composition, size and flexibility with a variety of lipid molecules and further surface modification. One of the most important advantages of liposomes is their ability to fuse with cell membrane and release their contents into cytoplasm, which makes them suitable intelligent carrier systems for targeted delivery. The simplest liposome is composed of a lipid bilayer surrounding a hollow core with a diameter of 50-1000 nm. The therapeutic molecules can be loaded into this hollow core for delivery (Luecuta, Liggins, Watson & Burt, 2010). Depending on the number of bilayers, they are classified into three basic types namely:

multilamellar, small unilamellar and large unilamellar. Multilamellar vesicles consist of several lipid bilayers separated from one another by aqueous spaces. In contrast, unilamellar vesicles consists of a single bilayer surrounding the entrapped aqueous space. These structural properties allow them to carry both hydrophobic and hydrophilic molecules. Hydrophilic molecules can be carried in the aqueous interior of the liposome, while hydrophobic molecules can be dissolved in the lipid membrane (Oberholzer et al., 2002). Additionally, more than one type of drug can be loaded either within two compartments (lipid and aqueous) or several aqueous layers of multilamellar liposomes. This also allows different drug molecules to be released in sequence with dissociation of layers from the outer shell to the inner core. The neutral or positively charged small liposomes have higher circulation time when compared to large, unmodified liposomes. Surface modifications can be obtained by either coating it with a functionalized polymer that improve targeted delivery and increase their circulation time in biological system (Gabizon et al., 1994). The liposomes are investigated for a wide variety of therapeutic applications such as cancer diagnostic and therapy, vaccines, brain-targeted drug delivery and anti-microbial therapy.

2.4.2 Exosomes

Exosomes are cell membrane-like lipid bilayer vesicles which contain various substances including RNA, DNA, glycolipids and proteins. They are naturally formed and secreted by various cell types. They are endosome-derived extracellular vesicles with a size of 30-150 nm and usually exists in different body fluids such as saliva, blood, urine and breastmilk. Exosomes play an important role in intracellular communication by transferring various compounds in physiological mechanisms such as immune response, neural communication, antigen presentation in diseases like cancer, cardiovascular disease, diabetes and inflammation. Since

these vesicles can be isolated from a patient's bodily fluids, allogenic exosomes have an advantage over the immune system, which can easily protect the cargo from rapid clearance and improve the drug delivery to targeted sites (Batrakova et al., 2015).

Over the past few years, considerable efforts have been made to develop exosomes as novel nanoscale delivery vesicle due to its several characteristics such as biocompatibility and biodegradable. This actually means that they do not elicit acute immune reactions, they have little or no toxicity and they carry the required fusogenic properties that is to say they contain low-uptake machinery, high specificity to target cells and are smaller in size making exosomes attractive nanocarrier drug delivery systems. In addition, exosomes have the tendency to accumulate more in tumor cells than in normal cells (Tan, Rajadas & Seifalian, 2012); (Milman, Ginini & Gil, 2019); (Arun, Nagavendria & Narendar, 2021).

2.4.3 Solid Lipid Nanoparticles (SLN)

They are aqueous colloidal dispersions comprising of a lipid matrix that is solid at room temperature. Surfactants improve their stability, whereas the choice of lipid affects the drug delivery characteristics. The size of SLNs varies from 10-1000 nm depending on the production approach (Dejesus, Gonzalez, Alphenaar & Atyabi, 2015). Solid -lipid nanoparticles as a sub-category of lipid carriers, can encapsulate very high amounts of lipophilic drugs as well as hydrophilic drugs and nucleic acids, making them versatile drug delivery vehicles (Ezzati, Shavanfar, Dinavand & Atyabi, 2015). SLNs can be decorated or loaded with various moieties including antibodies, magnetic nanoparticles, P^H sensitive lipids/polymers to modulate targeted delivery and stimuli-responsive drug release. They are shown to be effective carriers for cancer, pulmonary and oral drug delivery purposes

2.4.4 Nanocrystalline particles

Nanocrystalline particles or nanocrystals are carrier-free drug particles with a crystallite size of only a few nanometers. Its formulations are widely prepared for poorly water-soluble drugs suffering from limited bioavailability and absorption as a highly cost-effective approach. The size reduction is a suitable way to enhance the bioavailability of agents, where the dissolution velocity is the rate-limiting step. The crystalline structure leads to an increased overall surface area and hence increases velocity. This characteristic improves the solubility which is important especially when the therapeutic index of the agent is limited due to absorption problems. Nanocyrstalline particles enable the quick absorption of therapeutic agents due to their fast dissolution. This offers an advantage for agents that need to work fast. By modifying the nanocrystal surface, it is possible to achieve a prolonged or targeted release, allowing for the use of therapeutic agents in low doses and decreasing side effects. (Junnyapraset et al., 2015)

2.5 Synthesis of Medicinal Nanoparticles

There are two basic ways to synthesize nanoparticles: the **top-down approach** and the **bottom-up approach**. The top-down approach involves production of nanoparticle from macro sized materials while the bottom-up approach involves synthesis of nanoparticles from atoms. Between the two methods, the bottom-up approach is more popular and developed. It generally relies on synthesis pathways of two categories: **simultaneous** and **successive** methods. The simultaneous method demands for precursor materials of the metal of interest. The material can be bimetallic, trimetallic or alloy clusters in the same reaction, while the successive method involves the growth of particles by reducing metal ions over the surface of another metal core (Srinoi et al., 2018). There are also physical, chemical and biological based method of synthesis of nanoparticles.

2.5.1 Physical method of Synthesis

Under physical method of synthesis, there are sputtering, thermal decomposition, radiolytic method and sonochemical synthesis.

In sputtering, nanoparticles are created by bombarding the target metal with high energy. It involves three steps: migration of atoms from the surface of materials, nucleation, growth of nanoparticles and absorption onto another material in an electric field (Panda, 2010).

In thermal decomposition, nanoparticles are synthesized based on temperature. Synthesis of transition metal nanoparticles such as Iron (Fe), Nickel (Ni) and Cobalt (Co), requires high temperatures because these metal nanoparticles are not stable at room temperature. The method is also applied for metals that have low reduction potential or difficult reduction characteristics (Kin-Hung et al., 2020).

In radiolytic method, metal nanoparticles ae fabricated by irradiation which is called radiolytic synthesis. In this case, the gamma (γ)ray or electron beam is used to reduce metal ions in soluble precursors thus forming metal nanoparticles. This method can produce alloy nanoparticles that are not stable when created by thermal decomposition. The type of alloy nanoparticle created depends on the dose of irradiation. A low dose can lead to the creation of core-shell alloy nanoparticles whereas a higher dose controls the making of mixed alloy nanoparticles (Calineseu et al., 2014). The difficulty of the radiolytic method is in directing the nanoparticles' shape, however, irradiation-based techniques are low-cost, environmental-friendly and thus show promise for large applications.

In sonochemical synthesis, the method is based on ultrasound. In solution, ultrasound can cause high temperature or high pressure. Due to the increased temperature, small metal nanoparticles are created at a rapid reaction rate.

2.5.2 Chemical Method of Synthesis

In chemical method of synthesis, there are chemical reduction, electrochemical synthesis, hydrothermal, chemical precipitation and other chemical methods.

Chemical reduction method is used for producing bi/tri-metallic alloy nanoparticles through the reduction of appropriate precursors to the zero-valent state. It involves two phases, reduction and growth. The reduction process occurs sequentially, the first is that the metal precursors owing to the highest redox potential, precipitate to form the core, followed by the second and possibly the third precursor being deposited as a shell. Organic solvents are used to prevent agglomeration and maintain the stability of nanoparticles in the solution phase. The advantages of the core-reduction technique include simplicity of steps and versatile application but still has the disadvantage of presence of impurities.

Electrochemical synthesis uses electricity as the main source of composite reactions. This method is mostly used in industrial applications. An electrical field is created by two electrodes. Reduction occurs at the metallic anode or with the anode itself dissolved in solution along with the metal precursor and new metallic nanoparticles are formed at the cathode. For stabilization of fresh metal particles, a stabilized chemical has to be included. This method has advantages of controlling the nanoparticle size, imparting high purity, being environmentally friendly and cost effective (Ferrando, Jellinek & Johnson, 2008). This technique is applied in large scale manufacture of many bimetallic and trimetallic alloy particles (Gholivand et al., 2015)

In hydrothermal synthesis, nanoparticles are synthesized in high-temperature aqueous solutions at a high vapour pressure. Though this method allows monitoring of the nanoparticle growth, their physical and chemical properties, its disadvantages include high-temperature conditions and high-cost equipment. Many bimetallic nanoparticles such as nickel ferrite ($NiFe^2O^4$), and Co-Ni nanoparticles are created using the hydrothermal method (Rak & Brenner, 2019).

Chemical precipitation involves the formation of solids from a solution by creating supersaturated condition or by converting the soluble material into an insoluble form through P^H change, elctrooxidising potential or adding of a precipitation reagent. A typical chemical precipitation method contains four stages: flocculation, sedimentation and solid –liquid separation and filteration. It is also useful in large scale production of nanoparticles.

Other chemical methods that are included involve synthesis in homogenous liquids such as water or organic solvents. There are other synthesis methods that use the gas phase or heterogeneous phases such as sol-gel and micro-emulsion. There are also methods by which metal nanoparticles are synthesized in a gaseous environment such as the selective catalytic reduction method and flame spray pyrolysis. In flame spray pyrolysis method, a metal precursor solution that is sprayed is reduced by temperature, turning into a metal particle (Nunes et al., 2019). In the selective catalytic reduction technique, the reaction changes nitrogen oxides using a gaseous catalyst such as urea and ammonia. The sol-gel approach involves the basic steps of hydrolysis, condensation and drying. There are two types, aqueous sol-gel in which the solvent is water and nonaqueous sol-gel in which the solvent is an organic solvent. This method is simple, economical and can be processed at low temperature. The micro-emulsion method is a system comprising three components: a minor droplet (dispersed phase), an immiscible solvent (continuous phase), and a surfactant that covers the droplet. Depending on the properties of the dispersed phase, continuous

phase and the hydrophilic-lipophilic balance value of the surfactant, there are many types of micro-emulsions such as water-oil, oil-water and water-triton X-100 among others. The metal nanoparticles are synthesized inside droplets that can be designed to the desired size and composition. This method has been broadly applied in the synthesis of bimetallic and trimetallic alloy nanoparticles.

2.5.3 Biological methods of nanoparticle synthesis

Many approaches for nanoparticle synthesis have been discovered and improved since the development of nanotechnology. (chemical methods that are based on the use of hazardous chemicals while physical methods are based on enormous energy and high temperature and they eventually form nanoparticles with limited properties. To overcome these disadvantages, green synthesis approaches such as those based on microwave, electrochemical, hydrothermal and sonochemical methods have been developed. Another green synthesis method that has progressed recently is based on biological sources such as plants, microorganisms, industrial and agricultural wastes. This method has been applied to a large extent in nanoparticle production and has also been used for fabricating bimetallic or trimetallic alloy nanoparticles (Gholivand et al., 2015)

In using microorganisms to produce nanoparticles, micro-sized organisms including bacteria, fungi, yeasts and even viruses have been considered as nano factories to produce nanoparticles because of their ability to accumulate and detoxify heavy metals through various reductase enzymes. The metal reduction can be carried out in the extracellular or intracellular environment. The genes, proteins, enzymes and biomolecules of the microorganisms play roles as reducing factors. Bacteria such as *Escherichia coli, Salmonella typhi, Listeria monocytogenes* among others have been used to create alloy nanoparticles.

Recently, plants have been explored as an option for the green synthesis of nanomaterials. This involves the application of various plant organs such as root, stem, leaf, seed, fruit peel, flowers and their extracts to manufacture nanoparticles. This method is eco-friendly and stable and the synthesized nanoparticles have great potential use in biomedical and environmental applications. It is proposed that plant constituents such as protein, amino acids, organic acids, polysaccharides, secondary metabolites like polyphenols, flavonoids, alkaloids, terpenoid and heterocyclic compounds all play roles as reducing agents and stabilizing factors. Monometallic nanoparticles as well as metallic alloy nanoparticles have been manufactured using plant-based approaches, for example, leaf extracts of *Canna indica* have been used to produce Ag-Ni, Ag-Co, Pt-Cu, Au-Ag, Ag-Cu and Zn-ag nanoparticles. Also, leaf extracts of *Moringa oleifera* have been used to produce Au-Ag-Sr nanoparticles. Algae, which is a small eukaryotic organism is also used for the synthesis of alloy nanoparticles, for instance Phaeodactylum *tricornutum, Chlamydomonas reinhardtii and Spirulina platensis* were utilized to prepare Cds and Au-Ag nanoparticles (Rao & Rennatheur, 2017).

In green synthesis generally, the biological extracts are mixed with metal salts solution (Amit, Yusuf & Utama, 2013). The bio compounds which include phytochemicals such as alkaloids, phenolic compounds, terpenoids, enzymes, co-enzymes, proteins and sugar will reduce metal salts from positive oxidation state to zero oxidation state (Anupam et al., 2019). Size to size distribution of metallic nanoparticles depends on the bio compounds present in the extract. Availability of a strong reductant in the extract brings about fast reaction rate and favours the formation of smaller nanoparticles. In order words, in green synthesis of silver nanoparticles (AgNp), biological extracts in situ reduces silver salts (Ag^{+}) to metallic silver, Ag^{0}. In the process of nanoparticle synthesis, biological molecules not only reduce the metal salts but also cover the formed nanoparticles acting as reducing and capping agent (Anupam et al., 2019). This

capping is advantageous in the sense that it is multifunctional. It prevents agglomeration of the nanoparticle and reduces its toxicity if any (Demurtas & Perry, 2013) ; (Roy et al., 2013). if these coating agents which are the bio compounds show a characteristic towards what one wants to achieve like say antimicrobial, antifungal, antioxidant etc., a synergistic effect of the metal nanoparticle and the capped bio molecule may be found.

Though the ability of plant extract to reduce metal ions has been known, the use of live plants, plant extracts or plant tissues to reduce metal ions to nanoparticle has attracted considerable attention since the last 30 years. Live plants or plant tissues can be used for making nanoparticles, but the use of plant extract is relatively cheaper and simpler compared to whole plant or plant tissues. Also, the source of plant extract is known to influence the characteristics of the nanoparticles because different extracts contain different combinations and concentrations of the organic reducing agent (Mukunthan & Balaji, 2012). Drugs bound to the nanoparticles have been claimed to have more advantages compared to the conventional forms of the drugs (Wagner, Dullart, Bock & Zweck, 2006). The nano bound drugs have an extended half-life in vivo, longer circulation times and conveys a high concentration of the plant drug to the actual site needed (Sahoo, Parven & Panda, 2007). As the nano bound drug cannot circulate broadly, its side effects are reduced and high localized concentration achieved where needed. The nano bound drugs have the ability to penetrate into organs and tissues where needed because of their size and shape.

Agricultural and industrial wastes can also be used as source of nanoparticles.

In recent years, nanoparticles have been synthesized largely from agricultural and industrial wastes. Post-harvest wastes like fruit peel, rice husk and egg shells all form approximately 80% of the biomas on the field, and industrial wastes such as a stimber dust, sugarcane bagasse and

wild weeds including unwanted plants, herbs or shrubs that are usually burned can be used as biological sources for the green synthesis of nanoparticles. The use of these waste materials have benefits over physical and chemical methods which include reduction of using harmful chemicals, low cost, low energy and renewing waste material.

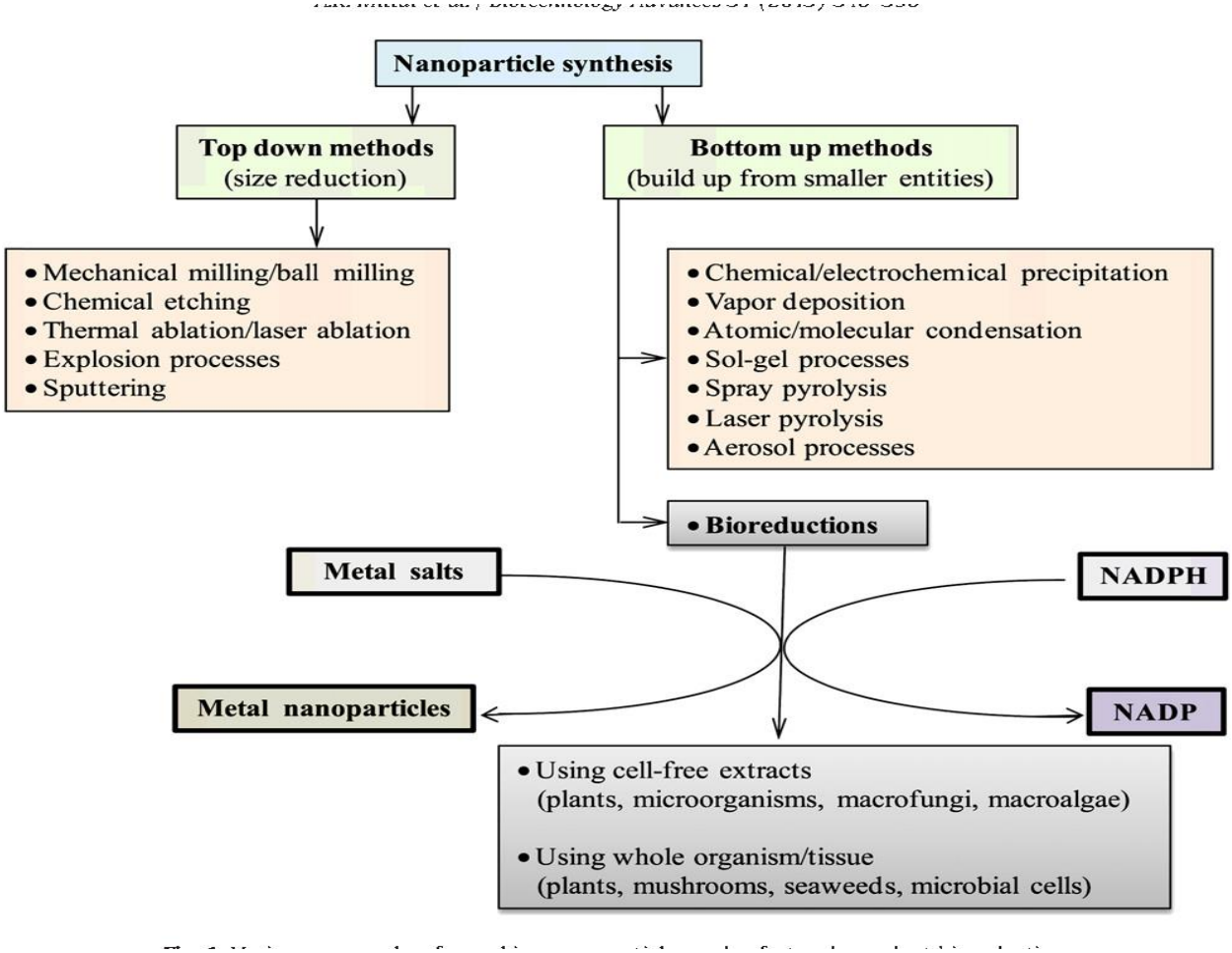

Figure 2.5: Approaches for nanoparticle synthesis and cofactor dependent bioreduction (Mittal, Christi & Banerjee, 2013)

Many monometallic nanoparticles were manufactured using citrus fruit peel extract, grape waste, mango peel, rice husk, sugar cane bagasse, eggshell, coconut shell and leaves. Typically, biological synthesis depends on P^H, temperature, pressure, time and protocol and has various advantages such as being ecofriendly, low-cost, safe and sample method that requires a short time. The biosynthesized nanoparticles are biocompatible and can be introduced directly into

biological and pharmacological applications without attaching to bioactive compounds. However, there is adequate need to check the effect of complicated parameters or complex constituents of plant organs on these synthesis methods.

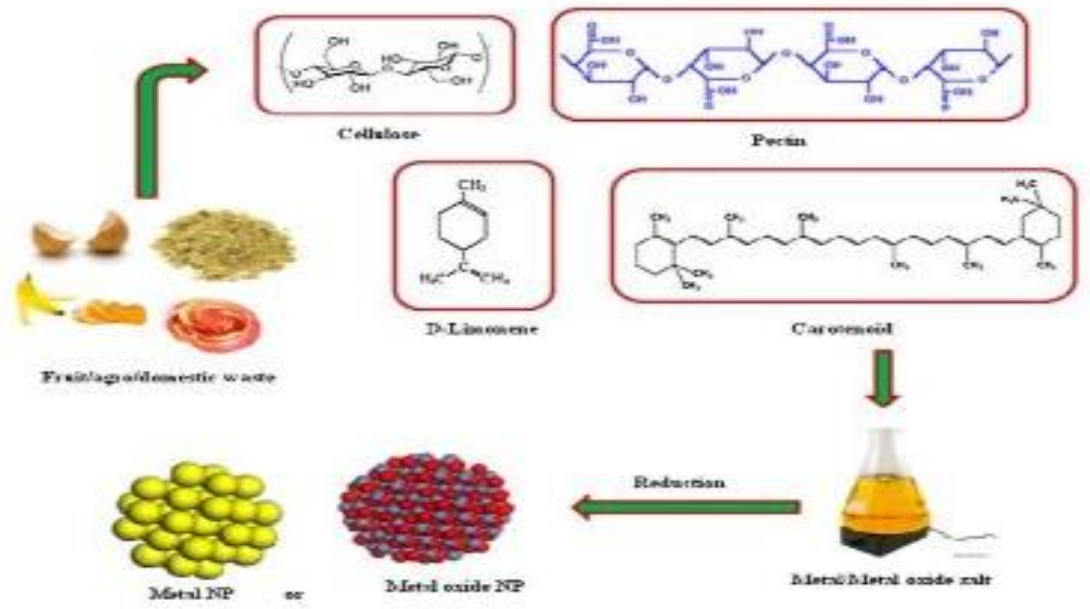

Figure 2.6: Synthesis of Nanoparticle Using Waste Materials (Sharma, Kumar, Kumar & Yadav, 2015)

In this instance, the biological synthesis was done using *Hibiscus sabdariffa*, a plant widely used in traditional medicine. It has been employed in the green synthesis of silver nanoparticles (AgNps). Studies have demonstrated the potential of *Hibiscus sabdariffa* extract as a reducing agent in synthesizing AgNps (Ajmal, Siddiqi, Alam & Waseem, 2018; Kumar, Das & Kumar, 2019).

Studies have also evaluated the cytotoxicity and biocompatibility of *Hibiscus sabdariffa* AgNPs using in vitro and in vivo models (Singh, Kumar, Kumar & Yadav, 2020; Rao *et al*., 2020). The results suggest that *Hibiscus sabdariffa* AgNps are relatively nontoxic and biocompatible, although further investigations are necessary.

Hibiscus sabdariffa AgNps have been explored for various applications, including wound healing, food preservation and antimicrobial packaging (Kumar, Kumar, Panthari & Choudhary, 2020; Patel, Sharma, Yadav & Singh, 2020). In a nutshell, *Hibiscus sabdariffa* AgNps have demonstrated potential antioxidant, antimicrobial and biocompatible properties, making them suitable for various applications. However, further research is necessary to fully understand their mechanisms of actions and potential toxicity.

2.5.4 Silver as an antimicrobial agent

Silver has long been used in medicine thousands of years back, for instance, silver was used as storage devices during historical periods while silver nitrate solution was directly used for wound healing during the second world war (Law et al, 2008). Before the advent of silver nanoparticle, silver was the main component in various creams for eczema and wound healing, scientists have long known that the metal is a potent antimicrobial agent that could further be reduced to nanoparticle in order to achieve an effective and efficacious agent due to its size and shape when further reduced.

The antimicrobial activity of *Hibiscus sabdariffa* AgNps has been investigated against various human pathogenic bacteria and fungi (Kumar, Kumar, Panthari & Choudhary, 2020; Patel, Sharma, Yadav & Singh, 2020). The results indicate that *Hibiscus sabdariffa* AgNps exhibit significant antibacterial and antifungal activity, making them potential agents for wound healing and food preservation.

2.5.5 Mechanism of antimicrobial action of Silver Nanoparticles (AgNps)

There has been an extensive research on the mechanism of antimicrobial action of silver nanoparticles, and three well defined mechanisms has been proposed:

(i) Cell wall and membrane damage

(ii) Intracellular penetration and damage

(iii) Oxidative stress (Dakal, Kumar, Majumda & Yadav, 2016)

Components of the cell wall and membrane exert different adhesion pathways for nanoparticles. Recall that one of the functions of the cell wall and membrane is to protect the integrity of microorganisms against environmental threat and to maintain homeostasis while still permitting the transport of nutrients inside the cell. Bacterial classification is based on the differences of the cell wall structures. Gram negative cell wall is thinner and contains lipopolysaccharide while gram positive cell wall is typically thicker and contains peptidoglycan. Most silver nanoparticles exhibit higher antibacterial activity against gram negative bacteria than gram positive bacteria because its peptidoglycan provides natural barrier which prevents penetration of nanoparticles. The negative charge of the lipopolysaccharide in gram negative bacteria facilitates adhesion of the nanoparticle (Gopinath et al 2017) ; (Feng et al 2000) ; (Beveridge, 1999). The interaction between AgNps and microorganisms starts with adhesion of AgNps to cell wall and membrane which is based on electrostatic attraction. Upon such an attraction and interaction, morphological changes in the membrane structure are triggered by the nanoparticles, leading to disruption of the membrane permeability and respiratory functions through membrane depolarization, and finally disruption of the cell integrity and death. Other mechanisms of action are as follows:

1. Release of silver ions: Silver nanoparticles can release silver ions which can interact with microbial cell membranes disrupting their structure and function, it will bind to DNA inhibiting replication and transcription and inhibit enzyme activity by disrupting metabolic processes.

2. Interference with nutrient uptake: Silver nanoparticles can inhibit the uptake of essential nutrients by microbial cells, leading to starvation, growth inhibition and reduced viability

3. Modulation of microbial signaling pathway: Silver nanoparticles can interact with microbial signaling pathways, leading to disruption of quorum sensing and biofilm formation.

Depending on the degree of membrane damage, AgNps can penetrate inside the cell and affect crucial and vital functions of the cell by interacting with DNA and Proteins (Gogoi et al., 2006). One of the proposed mechanisms for antimicrobial activity of AgNps is based on silver ion release from the nanoparticles which has an adverse effect on both DNA and Protein (Hsueh et al., 2015) (Bondarenko et al, 2013). Recent research showed that silver ion lead to the transformation of bacterial DNA from the natural relaxed state to a condensed state in which the DNA loses its replication ability (Feng et al., 2000). X-ray analysis demonstrated the Sulphur existence which indicates that silver ions reacted with thiol group of proteins and led to inactivation of enzymatic activity. It has also been shown that silver ions are physically attracted to DNA and interact with nucleoside part of the nucleotide and as a result, the base pairing between the complementary strand is disrupted by breaking the hydrogen bond (Hsueh et al., 2015) ; (Rhaban, Divsalar, Saboury & Golestani, 2010) ; (Pramanik et al., 2016).

2.5.6 Free Radicals and Oxidative Stress

Oxidative stress is a normal cellular process involved in many aspects of cellular signaling, however excessive oxidative stress can be harmful.

Reactive oxygen species (ROS) are oxygen containing molecules with a strong redox potential. ROS production and antioxidant capacity in the cell are balanced under normal circumstances.

However, in the case of imbalance between antioxidant mechanism and excessive production of ROS, the redox balance of the cell favours oxidation leading to oxidative stress (Schieber & Chandel, 2014). Previous studies reveal that nanoparticle challenge triggers cellular oxidative stress (Ravichandran et al., 2018); (Verma et al., 2017); (Fu, Yuan & Gao, 2015). In other to overcome the stress, cells exhibit different protective responses which includes enzymatic or non-enzymatic defense mechanisms. Note that when the oxidative stress overcomes the defense mechanism, the cell wall and biomolecules such as protein, lipid and DNA are subjected to damage caused by ROS and free radicals such as hydroxyl radical (OH^-), hypchlorous acid (HOCL), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Previous studies revealed oxidation induced DNA fragmentation following exposure to metal oxide nanoparticle (Sen et al., 2013); (Slavin, Asnis, Hafeli & Bach, 2017) ; (Yang et al., 2009). The ROS mediated antibacterial activity of AgNps against multidrug resistant *E-coli* and *S. aureus* were evaluated and it was found that ROS generation significantly contributed to the antibacterial activity (Das et al., 2017). Hence, the increased antibacterial activity in the presence of enhanced ROS production demonstrated that AgNp-induced ROS production is vital for antimicrobial activity. In other words, AgNp generates oxidative stress in the microrganisms together with the antimicrobial action and the resultant effect will lead to damage of vital biomolecules and consequently death of the organism.

Free radicals are atoms or molecules with unpaired electrons and thus are unstable. The unstable radical has the tendency of becoming stable by electron pairing with macromolecules such as protein, lipids and DNA, thus causing protein and DNA damage (Gilgun-Sherki, Rosenbaum, Melamed & Offen, 2002). Accumulation of free radicals brings about oxidative stress which causes oxidative damage. The body is under constant attack from oxidative stress and this causes damage to cells, proteins and DNA (Jessie, Sivanandbam & Manivasagam, 2016).

Free radicals are often associated with human diseases some of which may include cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease and many others. Causes of aging may also be attributed to gradual accumulation of free radical damage. Free radicals can be generated from daily activities and substances like fried foods, alcohol, tobacco, smoke, pesticides and air pollutants. They are also natural byproducts of chemical processes such as metabolism. Free radicals once formed can bring about chain reactions. The first free radical pulls an electron from a molecule, destabilizes the molecule and turns it into a free radical and this effect can go on and on which will eventually disrupt and damage the whole cell. It may lead to broken cell membranes and change in the structure of a lipid. The damaged molecules may undergo mutation and grow tumors as well as having changes in the DNA code.

All radicals share some common properties due to their unpaired electrons such as having unique species and being present under special and limited conditions. They are highly reactive and very unstable and can donate or accept an electron from other molecules. They behave as oxidants or reductants. Their common examples are superoxide anion radical (O^{2-}) hydrogen peroxide (H_2O_2), hydrogen oxide (OH), nitric oxide radical (NO) and peroxynitrite radical (ONOO). Free radicals can be generated internally in the mitochondria, through inflammation, exercise, phagocytosis and peroxisomes while they can be generated externally through the following sources: environmental pollution, cigarette smoke, radiation, drugs and pesticides, and ozone layer.

Superoxide anion is a negatively charged single radical resulting from monoclonal reduction of molecular oxygen that acquires an electron during a reaction that requires energy. This reaction occurs in the presence of NADPH oxidase enzyme during phagocytosis or mitochondria cytochrome oxidase activity during cellular respiration process (Pambuk et al., 2019).

Hibiscus sabdariffa AgNps have exhibited antioxidant activity as evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2[1]-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays (Singh, Kumar, Kumar & Yadav, 2019; Rao et al., 2020).

The antioxidant activity of *Hibiscus sabdariffa* AgNps has been attributed to their ability to scavenge free radicals and inhibit oxidative stress (Singh, Kumar, Kumar &Yadav, 2020)

2.6 Characterization of Nanoparticles

Nanoparticles are generally characterized by their shape, size, surface area and dispersity. The common methods of characterizing nanoparticles make use of Uv-visible spectrophotometry, Dynamic light scattering (DLS), Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), powder x-ray diffraction (XRD) and energy dispersive spectroscopy (EDS) (Feldheim &Foss, 2002 ; Sepur, 2008; Shahverdi, Shakibaje and Nazari, 2011; Mittal, Christi & Banerjee, 2013).

In Uv-visible spectrophotometer, light wavelengths in the 300-800nm are generally used for characterization of various metal nanoparticles in the size range of 2-100nm (Feldheim & Foss, 2002).

Spectrophotometric absorption measurements in the wavelength ranges of 400-450nm (Huang & Yang, 2004) and 500-550nm (Shankar, Rai, Ahmad & Sastry, 2004) are used in the characterization of silver and gold nanoparticles respectively.

The dynamic light scattering (DLS) is used to characterize the surface charge and size distribution of particles suspended in liquid (Jang et al., 2009).

Scanning electron microscopy and transmission electron microscopy are used for morphological characterization at the nanometer to micrometer scale (Schafer, Hohonester, Trugler & Hofer, 2009). Transmission electron microscopy has a 1000-fold higher resolution compared to Scanning electron microscopy.

FTIR spectroscopy is usually used to detect the surface chemistry of the nanoparticles, organic functional groups and other surface chemical residues can be detected using FTIR.

XRD is used mainly for phase identification and characterization of the crystal structure of the nanoparticles. It is a versatile technique used commonly in the field of nanotechnology to characterize and acquire accurate information regarding the composition, crystal structure and crystalline grain size of the nanoparticle. When x-ray penetrates into the nanomaterial, the resulting diffraction pattern is compared with standard to obtain structural information while the elemental composition of metal nanoparticles is established using EDS (Strasser et al., 2010).

2.7 Mechanism of increased efficacy of nanoparticles in medicine and therapeutics

Nano drugs show higher oral bioavailability because they exhibit typical uptake mechanisms of absorptive endocytosis. Nanoparticles reportedly help in preventing drugs from being tarnished in the gastrointestinal region and help the delivery of sparingly water-soluble drugs to their target location (Javanta, Gitishree & Hanseung, 2018). These nanoparticles stay in the blood circulatory system for a prolonged period of time to enable the release of the specified dose of drugs. They cause fewer plasma fluctuations with reduced adverse effects (De villiens, Aramwit & Kwon, 2008). Due to their small sized nature, they penetrate in the tissue system and facilitate easy uptake of drugs by the cells. They also permit an efficient drug delivery and ensure immediate action at the targeted location. The uptake of nanoparticles by cells is much higher

than that of large particles, hence they directly interact to treat the diseased cells with improved efficiency and reduced or negligible side effects.

The efficacy of these nanoparticles as drug delivery vehicles varies depending on the size, shape and other inherent biophysical/chemical characteristics. The green chemistry route of designing nanoparticles loaded with drugs is widely encouraged as it minimizes the hazardous constituents in the biosynthetic process and lessens the side effects of the medications (lam et al., 2017). Adjustments in nanoparticle size, shape, hydrophobicity and surface changes can further enhance the bioactivity of these nanomaterials. Nanoparticles make use of **site-specific** and **target-oriented** mechanisms of drug delivery.

2.7.1 Site Specific Mechanism of Drug Delivery

In site specific targeting, there are **passive and self-delivery** mechanisms of drug delivery. In passive delivery, drugs are incorporated in the inner cavity of the structure mainly through hydrophobic effect. When the nano materials are targeted to a particular site, the intended amount of the drug is released because of the low content of the drug which is encapsulated in a hydrophobic environment (Lu et al., 2016).

Also, in self-delivery, the drugs intended for release are directly conjugated to the carrier nanostructure material for easy delivery. Here, the timing of release is necessary and very important for if the drug dissociates very quickly from its nano carrier without reaching the target site, it will reduce its bioactivity and efficacy. This is to say that the timing of release is very crucial so that the drug with its nano carrier will dissociate at the target site at the exact time required, so that the efficacy will be maximally achieved.

In all mechanisms, the main targets in the body are the receptors on cell membranes and antigens or proteins on the cell surfaces (kumara et al., 2012).

The mechanism of action of nano-extracts, such as *Hibiscus Sabdariffa* silver nanoparticles in exerting enhanced antioxidant and antimicrobial properties can be explained further as thus:

Increased surface area: Nano extracts have a larger surface area to volume ratio, allowing for more effective interaction with free radicals.

Improved bioavailability: Nano extracts can penetrate deeper into cells, enhancing their ability to neutralize free radicals.

Enhanced reactivity: Nano extracts can donate electrons more easily, neutralizing free radicals and terminating oxidative chain reactions.

Synergistic effects: Nano extracts may exhibit synergistic effects with other antioxidants and other antimicrobials amplifying their overall antioxidant capacity and enhancing their efficacy against microorganisms.

Release of silver ions: Silver nanoparticles can release silver ions, which can interact with microbial cell membranes disrupting their structure and function, bind to DNA inhibiting replication and transcription. It can also inhibit enzyme activity, disrupting metabolic processes and so on.

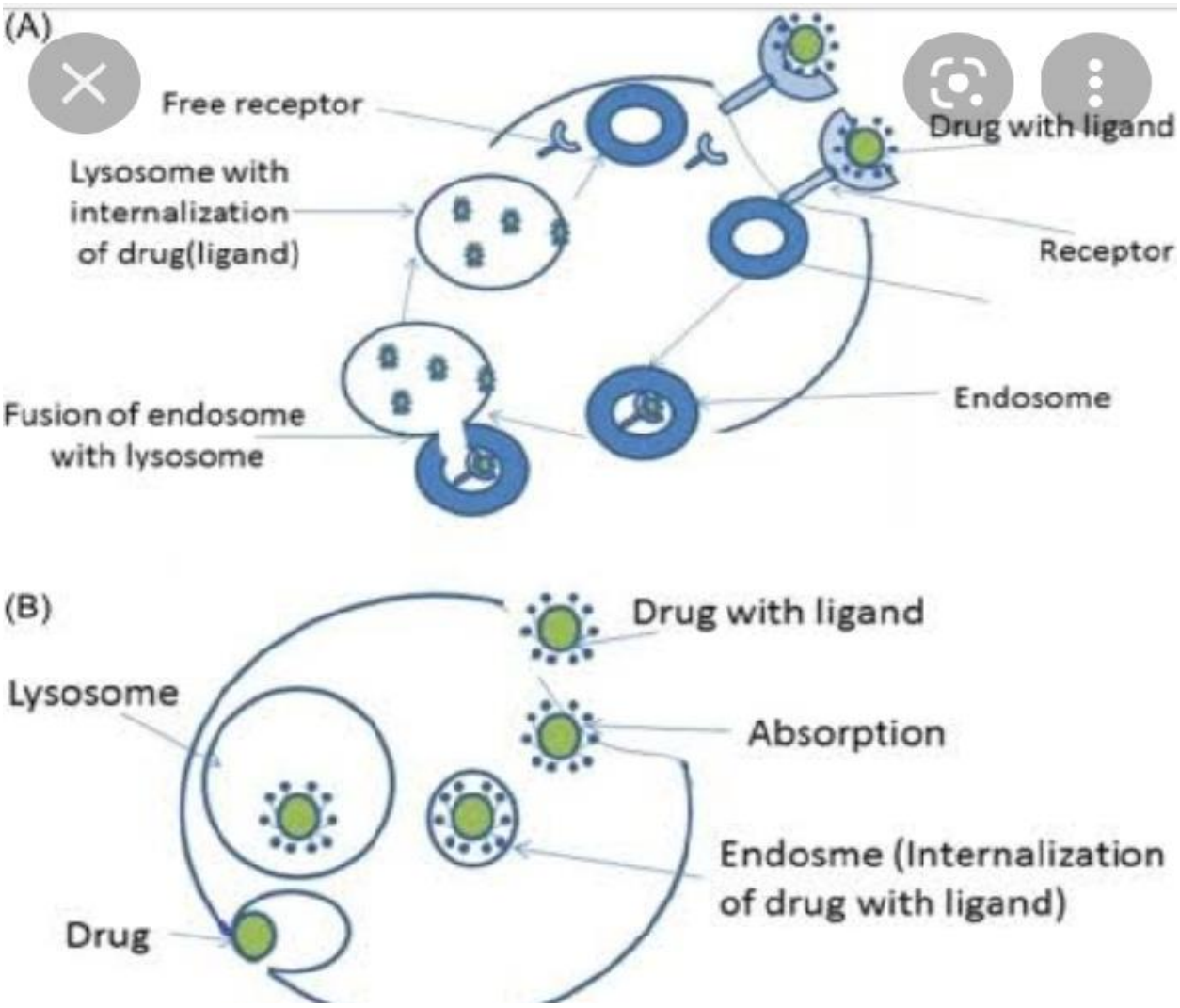

Figure 2.7 Site Specific Mechanism of Drug Delivery. (Tripti, Neeraj, Sharad & Sudheish, 2019**)**

2.7.2 Target-oriented mechanism of Drug Delivery

Targeting of drugs is classified into **active** and **passive.** In active targeting, moieties such as antibodies and peptides are coupled with drug delivery system to anchor them to the receptor structures expressed at the target site.

In passive targeting, the prepared drug carrier complex circulates through the blood stream and is driven to the target site by affinity or binding influenced by properties such as temperature, P^H, molecular size and shape.

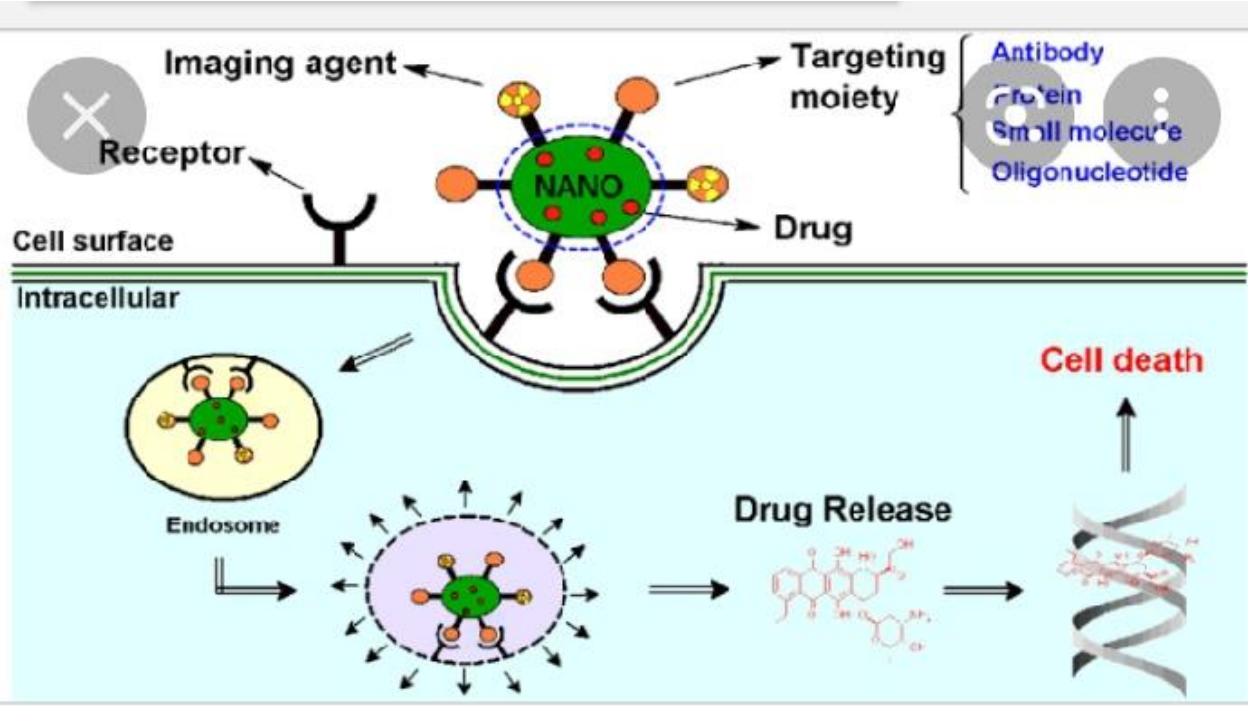

Figure 2.8: Target-oriented Mechanism of Drug delivery
(Tripti et al, *2019***)**

2.8 Medicinal Plants

Medicinal plants, also known as medicinal herbs, are plants that have been used for centuries to prevent, diagnose and treat various health conditions. These plants contain bioactive compounds such as alkaloids, glycosides, terpenes and much more that have therapeutic properties including anti-inflammatory, antimicrobial, antioxidant, anticancer, analgesic and antispasmodic effects.

Medicinal plants have been used in traditional medicine for centuries, with evidence of their use dating back to ancient civilizations such as in Nigeria, Egypt, China and India (World Health Organization (WHO), 2019). Traditional medicine involves the use of medicinal plants to treat various health conditions including fever, pain, digestive issues etc. (National Institutes of Health (NIH), 2020).

Medicinal plants have also contributed to the development of modern medicine with many drugs derived from plant sources (American Botanical Council (ABC), 2020). Examples of drugs

derived from medicinal plants include: aspirin from willow bark, taxol from *pacific yew*, artemisinin from *Artemisia annua*, digoxin from *Digitallis purpurea* and many more.

Medicinal plants are a valuable source of bioactive compounds for the development of pharmaceuticals (Mukherjee, Bhattacharya, Dasgupta & Roy, 2020). Examples include antibiotics such as penicillin from *Penicillium chrysogenum*) and anticancer drugs such as vinblastine from *Catharantus roseus*).

Medicinal plants are used in the development of cosmetics and skincare products such as Aloe vera for skin conditions, chamomile for soothing and calming effects.

Medicinal plants are also used as food supplements for example: ginseng for energy and vitality and echinacae for immune system support. There are so many medicinal plants in which the list is inexhaustible and *Hibiscus sabdariffa* is one of them.

2.9 *Hibiscus sabdariffa*: Origin

There are so many varieties of hibiscus but the popular variety that is edible and medicinal is the *Hibiscus sabdariffa*.

It is a herbaceous shrub belonging to the family of Malvaceae. It is believed to have originated in West Africa about 6000 years ago from where it may have been domesticated to other countries such as sudan for its seed and later for leaf and calyx production. In the 17^{th} century, the vegetable types were introduced to India and America. It is widely grown in tropics and subtropical regions of the world, such as in Africa, Central America, India, Caribbean, Brazil and Australia. It is a major crop of export in Sudan. It is also known as Roselle and in other English-speaking areas, it is called rozelle, sorrel, red sorrel, Jamaica sorrel, Indian sorrel, guinea sorrel, sour sour, queensland jelly plant, lemon bush and Florida cranberry. In India, it is called lambari,

gongura etc. while in Nigeria, West Africa it is called Zobo. *Hibiscus Sabdariffa* grows well in most soils that are well drained. The flowers are mainly hermaphrodite and are pollinated by insects. It requires a monthly rainfall of about three to four months before it grows.

Uses:

Roselle in Africa can be used as a vegetable and for preparation of beverage. The young shoots, leaves and calices are used as cooked vegetable in sauces. The sauces are eaten with tuber or cereal porridges or rice. The leaves and fresh green calices are used to make soup which is mucilaginous in texture. In some areas such as Senegal, the stewed calices of the green type are added as a condiment to rice dishes. In United States, the leaves and young shoots are also eaten raw in salads and the red fleshy calyx lobes are chopped and used in fruit salads. These uses are not normally common in Africa. In Cote d'Ivoire, the dried calyces ground to powder are normally used in sauces during dry season. The dried red calyces/leaves are used to prepare tea which could be taken either hot or cold after adding sugar and other stimulating ingredients such as ginger etc. The tea is usually a refreshing drink with a sour taste and is very popular from Senegal, Sudan, Egypt and other northern African countries where they call it 'karkade' while some West African countries such as Nigeria call it Zobo drink. The juice or drink is often sold chilled or frozen as the case may be. The dried calyses are used in western countries as a base for many herbal teas and as a source of red colourants. Roselle plants are often used as fodder for livestock following calyx harvest in West Africa. The oil of roselle seed is used for cooking in countries like Chad, China and Tanzania, even though the oil is claimed to contain some toxic substances and may better be used in soap and cosmetic industries. In Plateau region of Nigeria in West Africa, sorrel seeds are fermented to make cake used as 'sorrel meat' and the oil is used as ingredient for making paint.

Roselle extract has been used in folk lore medicine to treat colds, toothache, urinary tract infections and hangovers. Leaves are applied as a poultice to treat sores and ulcers. A root/leave/calyx decoction can be used as a laxative. Roselle tea can be used to suppress high blood pressure. In Senegal, the leave juice is used to treat conjunctivitis.

Roselle fiber is locally used in West Africa on a very small scale just like in Asia. The bast fiber is used to substitute jute for making twine, rope and sacks. The bast fiber together with the stem can sometimes be used in paper industry in the United States and in Asia. Roselle of recent has an ornamental value as a garden plant or flower. The decorative red stalks with ripe red fruits are exported to Europe for flower arrangements. Production of roselle leaves and calyces for domestic consumption in Africa is very common on local markets.

Nutritional Composition:

The nutritional composition of *Hibiscus Sabdariffa* varies between different studies, this might be as a result of different varieties, ecology, genetics, environmental and harvest conditions of the plant.

Previous studies had it that per 100 g of edible portion of roselle leaves contains 85.6 g of water, 180kj (43kcal) of energy, 3.3 g of protein, 0.3 g of fat, 9.2 g of carbohydrate, 1.6 g of dietary fiber, 213 mg of Calcium, 93 mg of phosphorus, 4.8 mg of iron, 4135 μg of β- carotene, 0.2 mg of thiamin, 0.45 mg of riboflavin, 1.2 mg of niacin and 54 mg of ascorbic acid. The composition of fresh raw calyces per 100 g edible portion contains86.2 g of water, 184 kj (44 kcal) of energy, 1.6 g of protein, 0.1 g of fat, 11.1 g of carbohydrate, 2.5 g of fiber, 160 mg of calcium, 60 mg of phosphorus, 3.8 mg of iron, 285 μg of β- carotene, 0.04 mg of thiamin, 0.06 mg of riboflavin, 0.5 mg of niacin and 14 mg of ascorbic acid. (Ismail et al, 2008). In addition to the above, the dried red calyces contain organic acids, polysaccharides, flavonoids and anthocyanin pigments known

as phytochemicals. (Eggensperger & Fakoya, 1996) ; (Muller & Franz, 1990). *Hibiscus sabdariffa* contains a high percentage of organic acids which include citric acid, hydroxycitric acid, hibiscus acid, malic and tartaric acid as the major constituients while having oxalic and ascorbic acid as the minor constituents. They also possess antispasmodic activity by relaxing the uterus and intestine strips invitro, which was also observed in rabbit aortic smooth muscle (Obiefuna et al., 1994), antioxidant activity by inhibiting formation of malondialdehyde content (Farombi & Fakoya, 2005) ; (Usoh, Akpan, Etim & Farombi, 2005), reduction of glutathione depletion, increase of the liver superoxide dismutase activity (SOD), catalase and glutathione and decreased malondialdehyde. (Mossalam et al., 2011). They also possess anthelminthic and bactericidal properties. The antihypertensive and cardioprotective effects of the tea made with roselle have been exhibited in various animal models and human clinical tests invivo (Ajiboye et al., 2011) ; (Adaramoye, Ogungbenro, Anyaegbu & Fafunsho, 2008) and in vitro (Ajiboye et al., 2011). The phenolic compound protocatechuic acid isolated from roselle flowers showed antioxidant, antitumour and hepatoprotective activities. Roselle extracts also showed antipyretic and anodyne properties in tests carried out with mice.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Extraction Materials:

Hibiscus sabdariffa flower, Whatman no1 filter paper, muslin cloth.

3.1.2 Chemical /Reagents

Absolute Ethanol, Deionized water, Silver Nitrate (Koch – Light Laboratories England) Chloroform, Diethyl Ether and Hexane, all of analytical grade from Merck Germany.

All other chemicals and reagents used were from varied sources and of analytical grade.

3.1.3 Glass Wares

Measuring Cylinder, Filteration Funnel, Conical Flask, Beakers, petri dishes, Test tubes, Round bottom flasks, Volumetric flasks, pipettes, microplates, cuvettes, spectrophotometer cells, spreaders, inoculation loops etc.

3.1.4 Equipment/ Apparatus

Digital pH meter (Labtech, India), Incubator, UV-visible spectrophotometer model D_{20} (Bausch and Laumb, Germany), Digital spectrophotometer model 390 (Turner®, USA), Rotary microtome, Digital Camera (Minolta, Japan), Hot air oven (Gallenpkam, England), Water bath (Grant, England), Digital weighing balance-Mettler PT 320 (Mettler-Wagen, Switzerland), Rotary shaker (Marrienfeld, Germany), Vaccum dessicators, Deep freezer (Freshpoint FDF-196),Bench centrifuge (Clay adams, USA), Automatic micro-pipettes (TECO® diagnostics,

USA), No 1 Whatman Filter paper, Magnetic Stirer (Searchtech Instruments, British Standards), Muslin Cloth, Gc-Ms 2010 QP Schimazu Japan. Rotary evaporator (Buchi Rotavapour – Switzerland),

3.1.5 Culture Media

Nutrient Agar (NA), Salmonella Shigella Agar (SSA), Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar (EMBA) and MacConkey Agar used for the characterization and identification of the test organisms were prepared according to manufacturer's specification.

3.2 Methods

3.2.1 Preparation of Plant Extract

Fresh flower of *Hibiscus sabdariffa* obtained from Eke Ukwu market Owerri, Imo State, Nigeria, identified by a taxonomist, were picked and removed of debris, dried at room temperature. The dried leaves were ground to fine powder using a mill (BL-335Kenwood) and stored in airtight container. Four hundred (400g) of the powdered flower were soaked in 2.0L, 80% ethanol. The solution was left to stand for 4 days with occasional agitation and later filtered through a qualitative filter paper (Whatman, No. 1). The crude leaf solution was rotor evaporated at 49^0C (Buchi Rotavapour, Japan) and the extract was stored in airtight container and kept in a refrigerator at -4^0C untill further analysis.

3.2.2 Preparation of Nano Extract

The method of Jayachandran et al, (2021) was used with slight modifications.

Fifty (50g) of the powdered *Hibiscus sabdariffa* flower were soaked in 250ml deionized water and heated in a water bath at 60 ^{0} C for 1 hour. It was allowed to cool, filtered with a muslin

cloth and re-filtered gradually with filter paper. Silver nitrate ($AgNo_3$) (1M) was added to the filtrate and heated again while stirring with a magnetic stirrer for 30 “min”. Solution was cooled and centrifuged at 1000 rpm for 15 minutes. The sediment was washed twice with deionized water and evaporated to dryness with a magnetic stirrer at controlled temperature. Sample was stored in the refrigerator at -4^0C untill further analysis.

3.2.3 GC-MS Analysis

GC-MS analysis was carried out with SHIMAZU Japan Gas Chromatography 5890-11 with a fused GC column OV 101 coated with polymethyl silicon (0.25 mm x 50 m) and the conditions are as follows: Temperature programming from 80 – 200^0C held at 80^0C for I minute, rate 5^0C/min and at 200^0C for 20 minutes. Flame Ionization Detector (FID) Temperature of 300 ^{0}C, injection temperature of 250 ^{0}C, carrier gas is Nitrogen at a flow rate of 1 cm^3/min, split ratio 1: 75. The column length was 30 m with a diameter of 0.25 mm and the flow rate of 50 ml/min. The eluents were automatically passed into the mass spectrometer with a detector voltage set at 1.5 kv and sampling rate of 0.2 seconds. The mass spectrum was also equipped with a computer fed mass spectra data bank, HERMCE Z 233 M-Z centrifuge Germany was used.

To identify the unknown phytochemical components present in the samples, their individual mass spectral peak value was compared with the database of National Institute of Science and Technology (NIST, 2014). Then identification was done by comparing the unknown peak value and chromatogram from GC-MS against the known chromatogram peak value from the NIST Library database. Subsequently, the details about their molecular formula, molecular weight, retention time and percentage content were also obtained.

3.2.4 Characterization of Nano extract

Characterization of the nano extract was carried out using these five methods: UV-Visible Absorbance Spectroscopy, Energy Dispersive Spectroscopy (EDX), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD).

3.2.4.1 UV-Visible Absorbance Spectroscopy

Uv- vis spectroscopy was performed by a Genesys Uv-Vis spectrophotometer (prove 300) with a serial number (2130315791) and a software version of ISW 1.5.1. The absorption spectrum was recorded from 200nm to 900nm. The samples were measured during different time intervals between 0 and 6 hours and after 24 hours of incubation. The mixtures were stored at room temperature in quartz cuvettes and in a day-night light condition in between measurements. Also, blanks (without silver nanoparticles) of an instrument detection limit (IDL) and a Limit of blank (LB) medium mixed in the ratio of 1:1 with the solvent specific for the stabilization type of the AgNps were measured. However, a visual analysis of the sample was performed in parallel.

3.2.4.2 Scanning Electron Microscopic Analysis

To determine the detailed morphological features of the synthesized AgNps, a Scanning Electron Microscopy (SEM)(JEOL.JSM-6380LV) (20kv) was used. The specimen used for the analysis was prepared by dispersing the powdered AgNps onto a SEM holder followed by gold (Au) coating aimed at enhancing the conductivity

3.2.4.3 Transmission Electron Microscopic Analysis

The transmission electron microscopy (TEM) was carried out for the confirmation of the shape of the particles and diffraction pattern. The powdered sample was loaded on carbon – coated

copper grids by drop cost techniques and analyzed by HR TEM Model Teenai G_2STWin (200kv). The size of the AgNps was calculated using the Scherrer equation:

Equation3.1: $L=K\lambda/\beta Cos\ \theta$; L= Nano Crystal, λ (Lambda) = wavelength; β (beta) = peak; K = shape factor (which ranges from 0.62 – 2.08 taken at 0.59); θ = taken at 2θ in the pattern.

3.2.4.4 Energy Dispersive Spectroscopic Analysis

The reduced AgNps were assayed for their purity and energy content by the energy dispersive x-ray spectroscopy with model EDS (JSM – IT500, Joel, Boston, MA, USA). The sample was dried on a copper grid coated with carbon. Electron beam were focused on the sample and emitted rays were detected using the EDX detector. Silver peaks were identified and elemental composition and purity was determined and compared with reference spectra verification.

3.2.4.5 X-Ray Diffraction Analysis

The synthesized silver nanoparticle was centrifuged at 10,000 rpm for 15 minutes and the pellets were redispersed in sterile double distilled water and centrifuged at 10,000 rpm for 10 minutes. The purified pellets were dried at 50^0C in an oven and analyzed by X-ray diffraction unit (XRD) (Pan Analytical, X-pert pro, Netherland). The X-ray diffraction (XRD) measurement of silver nanoparticles synthesized by leaf extracts was carried out using Cu-Kα radiation source in scattering range m(2θ) of 10-70 on the instrument operating at a voltage of 45 KV and a current of 40 mA. The presence, crystalline nature, phase variety and grain size of synthesized silver nanoparticles were determined by X-ray diffraction spectroscopy. The particle size of the prepared samples was determined by using Scherrer's equation as follows:

Equation 3.2: $D = K\lambda/\beta \cos\theta$

Where D is average crystalline size, β is line broadening in radians (full width at half maximum of the peak in radians), λ is wavelength of X-ray while θ is braggs angle. K is constant (geometric factor = 0.94)

3.2.5 Antimicrobial Analysis

The antimicrobial analysis of *Hibiscus sabdariffa* crude and nano extracts were carried out using Spectrophotometric assay.

3.2.5.1 Microscopic Identification of Bacteria isolates

Pure cultures of foodborne bacteria previously isolated from ready-to-eat pork meat were further subjected to routine microbiological screening to ascertain their purity, viability and identities was done using standard procedures. The characterization and identification of the isolates were done following the procedures below:

3.2.5.2 Preparation of media and diluents

Bacteriological media such as Nutrient agar (NA), Salmonella Shigella agar (SSA), Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar (EMBA) and MacConkey agar used for the characterization and identification of the test organisms were prepared according to manufacturer's specification (Sharma, Sethi & Gupta, 2000; Cheesbrough, 2000).

3.2.5.3 Characterization and identification of microbial isolates

Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals. The identities of the isolates were cross matched with reference to standard manuals for the identification of bacteria (Beishir, 1987; Buchannan and Gibbon, 2000).

The cultural characterization was done by inoculating the microorganisms on agar plates, which were incubated at 37 ^{0}C for 24 hours. The colony was observed for size, shape, colour and texture.

3.2.5.4 Microscopic characterization

Gram Staining Test

The Gram staining technique was used on the bacterial isolates as described by Cheesbrough (2000). A smear of the isolate was made on grease free glass slide with a drop of water and allowed to dry. The smear was fixed by mild heating, flooded with crystal violet and allowed to stand for 30 seconds. The crystal violet was rinsed off with water; Lugol's iodine was added and allowed to stand for 30 seconds. This was washed off with water and holding the slide on a tilt with a clothes pin slide, was flooded with ethanol for 5 seconds to wash off stain. It was counter stained with Safranin for 10 seconds and rinsed with water. The wet slide was allowed to air dry. A drop of oil immersion was added on the slide and viewed using X40 objective lens of the microscope. Fungal species were characterized and identified by wet mount method. This involves mixing the fungal sample with water or saline solution on a slide, covering with a coverslip and observing under microscope.

Spore Staining Test

The spore stain was used to confirm the presence of spores when indicated in the Gram stain. Isolates were heat fixed on a slide and flooded with 5% malachite green. It was sterilized for 3 minutes (without allowing it to boil), dried and cooled. It was then rinsed off and stained with Safranin for 30 seconds. This was rinsed, dried with filter paper and viewed under the

microscope using oil immersion tens. The positive spores showed green while the vegetative cells were stained pink.

Motility Test

This test was used to determine the motility of bacteria isolated. The test was carried out on a semi-solid agar medium in which motile bacteria swarm and gave a diffuse spreading growth. The medium was dispensed into test tubes, sterilized and allow to set in an upright position. It was then inoculated using an inoculation needle by stabbing it into the medium in the test tube. This was incubated at 37°C for 24 hours. Diffuse growth from the straight line of inoculation was recorded as positive result (Cheesbrough, 2000).

3.2.5.5 Biochemical Characterization of Bacteria Isolates

Microorganisms that were not identified by the colonial and microscopic characteristics were further subjected to biochemical tests described by Cheesbrough (2000) & Beishir (1987).

Catalase Test

Equation 3.3 $2H_2O_2 \rightarrow 2H_2O + O_2$

The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Catalase has one of the highest turnover numbers of all enzymes such that one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen in a second. Catalase activity was detected by adding the substrate H_2O_2 to an appropriately incubated (24 hours) tryptic soy agar slant culture. Organisms that produces catalase enzyme, breaks down hydrogen peroxide to water and oxygen, producing bubble in the reagent drop indicating a positive test. Organisms lacking the cytochrome system also lack the catalase

enzyme and were unable to breakdown hydrogen peroxide into O_2 and water and are catalase negative.

Coagulase Test

Coagulase is an enzyme that clot blood plasma by a mechanism that is similar to normal clotting. The coagulase test identifies whether an organism produces this exoenzyme. This enzyme clots the plasma component of blood. The only significant disease-causing bacteria of humans that produce coagulase are *Staphylococcus aureus.* Thus this test is a good indicator of *S. aureus.* In the test, the sample was added to rabbit plasma and held at 37ºC for a specified period of time. Formation of clot within 4 hours was indicated as positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result indicative of a non virulent strain. (ASM, 2020)

Oxidase Test

Oxidase test is an important differential procedure that should be performed on all gram-negative bacteria for their rapid identification. The test depends on the ability of certain bacteria to produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and ∞-naphthol. This method uses N, N-dimethyl-p-phenylenediamine oxalate in which all Staphylococci specie are oxidase negative. In the presence of the enzyme, cytochrome oxidase (gram negative bacteria) the N, N-dimethyl-p-phenylenediamine oxalate and ∞-naphthol react to give indophenol blue. Small portion of the bacterial culture is placed on a filter paper, 3 drops of oxidase reagent is added to the culture and allowed for 30 secs to observe for colour change. Colour change to dark blue, purple or black indicates presence of cytochrome c oxidase (CDC, 2020)

Sugar Fermentation/Oxidation

This test was used to differentiate between bacteria groups that oxidize carbohydrate such as members of Enterobacteriaceae. One milliliter (1ml) of 10% glucose, maltose, lactose, fructose, mannitol, and sucrose were separately under aseptic conditions transferred into duplicate tubes containing 9ml of sterile Hugh and Leifson's medium to obtain a final concentration of 1% of each of sugar. The tubes were stab-inoculated in duplicates while two uninoculated tubes serve as control. Vaseline was used to seal one set of the duplicate tubes and one control to discourage oxidative utilization of sugar. All tubes were incubated at 37^{o}c for 48h. After the incubation, they were observed for acid production in the culture. Yellow colouration indicated acid production in the open tubes only suggesting oxidative utilization of the sugar while acid production in the sealed tubes suggested a fermentative reaction (ASM, 2020)

Hydrogen Sulphide (H_2S) Test

The test isolates were aseptically inoculated into a tube containing triple sugar iron agar started by stabbing the agar to the bottom and streaking the surface of the slant. The inoculated tube was incubated at 37^{o}C for 72 h and was examined daily. Black precipitation and yellow colouration was checked for. Black precipitate indicated H_2S production and yellow colouration for sucrose, lactose and glucose fermentation.

Urease Test

Urease agar slant in McCartney bottle was inoculated with the bacteria isolate at 30^{o}C for 4 h and then overnight. A pink colour in the medium indicated a positive result.

IMViC Test

This test consists of four different tests. They are Indole production, methyl-red test, VogesProskaeur test and Citrate utilization test. This test is specifically designed to determine the physiological properties of microorganisms. They are especially useful in the differentiation of Gram-negative intestinal bacilli, particularly *Escherichia coli* and the *Enterobacter-klebsiella* group.

Indole Test

This test demonstrates the ability of certain bacteria to decompose the amino acid-tryptophan to Indole. The bacteria isolates were inoculated into the medium and incubated at 37°C for 48 h. At the end of incubation period, 3 drops of kovac's reagents were added and then shaken. A red colour ring at the interface of the medium denoted a positive result.

Methyl red (mR) and Voges-Proskaeur(vP) test must be considered together since they are physiologically related. Opposite test is usually obtained from the MR and VP tests, that is, MR+, VP-, or MR-, VP+.

Methyl red test was performed to demonstrate the capacity of different organisms to produce acid from the fermentation of sugar (dextrose). Methyl-red positive organisms produce a red colouration when five drops of methyl-red indicator were added into 48 h old MR-VP broth culture.

The Voges-Proskaeur test demonstrates the ability of organisms to produce acetoin from glucose metabolism. Some organisms metabolise glucose to produce pyruvic acid which is further broken down to yield Butane-diol and acetyl-methyl carbinol as intermediate products.

Into one milliliter of the culture, one milliliter of six percent alcoholic solution of alpha-naphtol and one milliliter of 16% KOH was added and stood for 20 mins. Development of red to pink colour indicates positive test.

Citrate Utilization Test

This is one of the several techniques used in the identification of Enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon. The test was carried out using Simmon's citrate agar.

The slopes of the media were prepared in bijou bottles as recommended by the manufacturers. A sterile straight wire was used to the slope with a saline suspension of the test organisms before stabbing the butt. The bottles were incubated at 35^{o}C for 48 h. Bright blue colours in the medium means positive test while no change in colour of medium indicated negative citrate test (Cheesbrough, 2000).

3.2.6 Preparation of Test Organism

Test organisms for standardization were sub-cultured on nutrient agar and nutrient broth at 37^{o}C for 24 h. Organisms were also grown on a slant for preservation (Gotep et al., 2009). Test isolates were standardized by McFarland method, using 0.5 MacFarland standard and mineral salt medium. McFarland solution consists of barium chloride and sulphuric acid.

3.2.7 Susceptibility Test

Susceptibility of the test isolates to the extract was done by agar well diffusion assay. Four wells of 6.25 mm deep were made with a sterile cork borer on Mueller Hinton Agar previously seeded with the 24 h old standardized cultures. The wells were filled with different concentrations (500,

250, 125 and 62.5 mg/ml) of the ethanol and aqueous extracts separately. The plates were incubated for 24 hours at 37^0C. After 24 hours, zone of inhibition around the wells were measured and recorded in millimeters (mm).

3.2.8 Minimum Inhibitory Concentration (MIC) Assay

The Minimum Inhibitory Concentration Assay is a technique used to determine the lowest concentration of a particular antibiotic needed to kill an organism. This assay is typically performed on planktonic (free floating) bacterial cells. To evaluate MIC, the procedure was done according to Atlas *et al*. (1995).

Serial dilutions of the extracts (representing different concentrations of 500, 250, 125 and 62.5 $mgml^{-1}$) were added to a growth medium (nutrient broth) in separate test tubes. These tubes were inoculated with the standardized test isolates and were incubated overnight. Broth tubes that appeared turbid were indicative of bacterial growth while tubes that remained clear indicated no growth. The MIC of the antibiotic/toxicant (plant extract) was the lowest concentration that does not show growth. This was confirmed using the spectrophotometer at 420 nm.

3.2.9 Minimum Bactericidal Concentration (MBC) Assay

Minimum Bactericidal Concentration is the least concentration of bacteria inhibited after 24 h incubation on nutrient agar. A loop full of the different concentrations (after spectrophotometric reading) were streaked on a freshly prepared surface dried nutrient agar and incubated overnight. Concentrations of growth after incubation was used to determine the MBC.

3.2.10 *In-vitro* Free Radical Scavenging Assay

3.2.10.1 Nitric oxide radical scavenging assay

The scavenging effect of extract on nitric oxide was measured according to the method of Marcocci *et al.,* (1994) as described by Alisi & Onyeze (2008). The assay was carried out in 4 ml volume consisting of varying concentrations of the extract (0 - 2000 µg/ml) and quercetin stock solution (0-1000 µg/ml) in phosphate buffer (pH. 7.2). To the tubes, 1ml of 5 mM sodium nitroprusside solution in phosphate buffer (pH. 7.2) was added and incubated at 29°C for 2 hours. Nitrite formed was measured by reacting 2 ml of the incubation solution with 1ml Griess reagent (equal volume of 1% sulphanilic acid and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride (NED). The absorbance was measured in a spectrophotometer at 550 nm. Inhibition of nitrite formation by extract or quercetin was calculated relative to the control which was void of extract or quercetin.

Equation 3.4:
$$\%\ Inhibition = \frac{Absorbance\ control - Absorbance\ test}{Absorbance\ control} \times 100$$

3.2.10.2 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging ability of extracts was measured by assessing 2-deoxyribose degradation by hydroxyl radicals generated from Fe^{3+} / ascorbate / EDTA / H_2O_2 system as described by Halliwell et al., (1987). The reaction mixture consisted of deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA(0.1mM), H_2O_2 (1mM), ascorbic acid (0.1mM), KH_2PO_4/K_2HPO_4–KOH buffer (20mM, pH 7.4) and the extract (0 – 3000 µg/ml) in a final volume of 1.0 ml. After incubation for 1 hr at 37^oC, deoxyribose degradation was measured as thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa, Ohishi & Yagi, *(*1975), as modified by Liu et al., (1990). The test set up was further treated with 1.5ml of 20% acetic acid, 1.5ml of

0.8% thiobarbituric acid (TBA), 0.2ml of 8.1% sodium dodecyl sulphate (SDS) and the mixture heated at 100^{o}C for 1 hr. This was cooled to room temperature and 2ml of trichloroacetic acid added, vortexed vigorously and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured in a spectrophotometer at 532nm wavelength. Inhibition of deoxyribose degradation which gives an indication of hydroxyl radical scavenging action and iron chelating activity (Lopes *et al.*, 1999) was calculated as follows:

Equation 3.5:
$$\%\ Inhibition = \frac{Absorbance\ control - Absorbance\ test}{Absorbance\ control} \times 100$$

3.2.10.3 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

The scavenging activity of extracts for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined according to the method of Blois (1958) as described by Velazquez *et al* (2003). The test consisted of 1.0ml extract (0 – 500 mg/ml) dissolved in methanol and mixed with 2 ml of 0.02 mg/ml DPPH (Fluka Chemie, Switzerland) in methanol. The set-up was incubated for 15 min at room temperature and its absorbance measured at 517 nm. Tannic acid (0 – 500 mg/ml) was used as positive control. The radical-scavenging activity was calculated as follows:

Equation 3.6:
$$\%\ DPPH\ radical\ scavenging = \frac{Blank\ absorbance - Absorbance\ test}{Blank\ absorbance} \times 100$$

The scavenging data was fitted into mathematical equations with highest correlation coefficient and used to evaluate IC_{50} (concentration causing 50% inhibition) values of the extract and standard.

Equation 3.7: IC50 = (50 x [DPPH]) / (1 +(10^(Log(EC50))).

3.2.10.4 Determination of Superoxide dismutase

This assay was carried out according to the procedure of Das, Chandra, Shrivastava and Sinha,2000. In the assay, the reaction mixture comprised of 1.1ml extract (0-4000µg/ml) in 50mM Phosphate buffer (pH=7.4), 0.075ml of 20mM L-Methionine, 0.4ml of 1%(V/V0 Triton X-100, 0.075ml of 10MM hydroxylamaine and 0.1ml of the sample was incubated at 30^{o}C for 5min. This was followed by the addition of 80ul of 50uM riboflavin and the tubes were exposed to incandescent light (200watts Lamp) for 10minutes. Thereafter, 1ml of 1% sulphanilamide and 1ml of 0.1% NED were added and the absorbance of the colour formed measured at 543nm.

The SOD radical-scavenging activity was calculated as follows:

Equation 3.8: $\%\ SOD\ scavenging = \frac{Absorbance_{Control} - Absorbance_{Test}}{Absorbance_{contol}}$

3.2.10.5 Total antioxidant capacity

The total antioxidant capacity of the extract was determined by using phosphomolybdenum as described by Prieto *et al.,* (1999). An aliquot of 0.4 mL of extract dissolved in methanol (1 mg/mL) was mixed with 4 mL of phosphomolybdenum reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a waterbath at 95º C for 90 minutes, cooled and the absorbance was measured at 695 nm. The total antioxidant activity of the sample was determined using a standard curve prepared for ascorbic acid. The total antioxidant capacity of the extract was expressed as mg of ascorbic acid equivalents (AAE)/g of extract.

3.3 Statistical analysis

Numerical data were presented for statistical analysis using Sigma plot software version 10 to fit free radical inhibition data and concentration into non-linear models to determine their relationship and IC_{50}.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

Phytochemical Screening result of the crude and nano extract of *Hibiscus sabdariffa* flower

The GC-MS result of the crude and nano extract of *Hibiscus sabdariffa* showed a total of Fifteen (15) biocomponents for the crude and a total of Fifteen (15) organic components for the nano extract.

Table 4.1.1 shows the bio components of the crude extract of *Hibiscus sabdariffa* obtained through GC-MS. A total of fifteen (15) bio-compounds were obtained in which 4-amino-1,2 naphtoquinone occurred highest at 33.82% abundance while Hexadecanoic acid methyl ester occurred least at 0.36% abundance. Their molecular weight ranges from 173 – 308. These compounds contained quinones, fatty acid methyl esters and benzamides which are responsible for their antioxidant and antimicrobial properties.

Table 4.1.1: Phytochemical Screening result of the crude extract of *Hibiscus sabdariffa*

Peak	Compound Name	Mol. Weight	Mol. Formula	% Composition	Retention time
1	Butanedioic acid, diethyl ester	174	$C_8H_{14}O_4$	1.83	2.936
2	Cyclohexanecarboxylic acid, 2-chloroethyl ester	190	$C_9H_{15}CLO_2$	13.52	3.136
3	Butanedioic acid hydroxy, diethyl ester	190	$C_8H_{14}O_5$	3.49	3.547
4	α-D-mannofuranoside	298	$C_{13}H_{24}B_2O_6$	10.09	3.713
5	Succinic acid, ethyl hexyl ester	230	$C_{12}H_{22}O_4$	2.58	4.216
6	Benzaldehyde, 2-fluoro-3,4-dihydroxy	156	$C_7H_5FO_3$	1.57	5.479
7	Aceticacid, 2 acetyl-5-methyl-isoxazolidin-5-methyl ester	201	$C_9H_{15}NO_2$	8.04	5.834
8	4-amino-1,2 naphtoquinone	173	$C_{10}H_7NO_2$	33.82	6.239
9	4-Trifluoromethyl benzamite	189	$C_8H_6F_3NO$	12.88	6.559
10	2H-1-Benzopyran-2-one,7-amino-4-trifluoromethyl	229	$C_{10}H_6F_3NO_2$	0.89	6.822
11	Pthalic acid, isobutyl 2-methylpent-3-yl ester	306	$C_{18}H_{26}O_4$	0.46	7.142
12	Hexadecenoic acid methyl ester	270	$C_{17}H_{34}O_2$	0.36	7.394
13	Palmitic acid, vinyl ester	282	$C_{18}H_{34}O_2$	4.33	7.731
14	9,12-octadecanoic acid, methyl ester	294	$C_{19}H_{34}O_2$	0.48	8.240
15	9,12-octadecanoic acid, ethyl ester	308	$C_{20}H_{36}O_2$	5.67	8.543

4.1.2: Phytochemical Screening result of the nano extract of *Hibiscus sabdariffa*

The table shows the phytochemical screening result of the nano extract of *Hibiscus sabdariffa* which showed a total of fifteen (15) organic compounds with Cis-Vaccenic acid occurring highest at 46.37% abundance while N-2-carbomethoxyvinyl methylamine occurred lowest at 0.93% abundance. Their molecular weight ranges from 84 – 356. These organic compounds contain polymers, lipids and other carbon-based components which are responsible for their antioxidant and antimicrobial properties including their cardiovascular benefits and nutritive values. The compounds obtained from the crude extract are quite different from the one obtained from the nano extract.

Table 4.1.2: Phytochemical Screening result of the nano extract of *Hibiscus sabdariffa*

Peak	Compound Name	Mol. Weight	Mol. Formula	% Composition	Retention time
1	Methylene chloride	84	CH_2CL_2	1.00	**3.536**
2	1,16-cyclocorynan-17-oic, 19,20 didehydro, methyl ester	322	$C_{20}H_{22}N_2O_2$	1.98	**5.508**
3	Allyl 2-ethyl butyrate	156	$C_9H_{16}O_2$	3.56	**6.119**
4	N-(2-carbomethoxyvinyl) methylamine	115	$C_5H_9NO_2$	0.93	**6.502**
5	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	1.13	**6.628**
6	Di-sec-butyl phthalate	278	$C_{16}H_{22}O_4$	2.93	**7.131**
7	n-hexadecanoic acid	256	$C_{16}H_{32}O_2$	30.92	**7.663**
8	Cis-Vaccenic acid	282	$C_{18}H_{34}O_2$	46.37	**8.520**
9	9-octadecanoic acid	282	$C_{18}H_{34}O_2$	1.21	**8.937**
10	6-octadecanoic acid	282	$C_{18}H_{34}O_2$	0.93	**9.497**
11	9-octadecanoic acid, 2,3-dihydroxypropyl ester	356	$C_{21}H_{40}O_4$	2.93	**10.017**
12	9-octadecanoic acid	282	$C_{18}H_{40}O_2$	2.06	**10.909**
13	17-pentatriacontene	490	$C_{35}H_{70}$	0.83	**12.561**
14	2,3-Dihydroxypropyl elaidate	356	$C_{21}H_{40}O_4$	2.05	**14.475**
15	9-octadecanoic acid 2,3-dihydroxypropyl ester	356	$C_{21}H_{40}O_4$	1.18	**16.470**

4.1.3 Spectrophotometric characterization of the nano extract of *Hibiscus sabdariffa*

Figure 4.1.3 shows the spectrophotometric result of the nano extract obtained during characterization using UV-Visible Spectrophotometer. The result showed maximum light absorption at a wavelength of 420 nm. This implies that the nanoparticle exhibited a surface plasmon resonance at 420 nm.

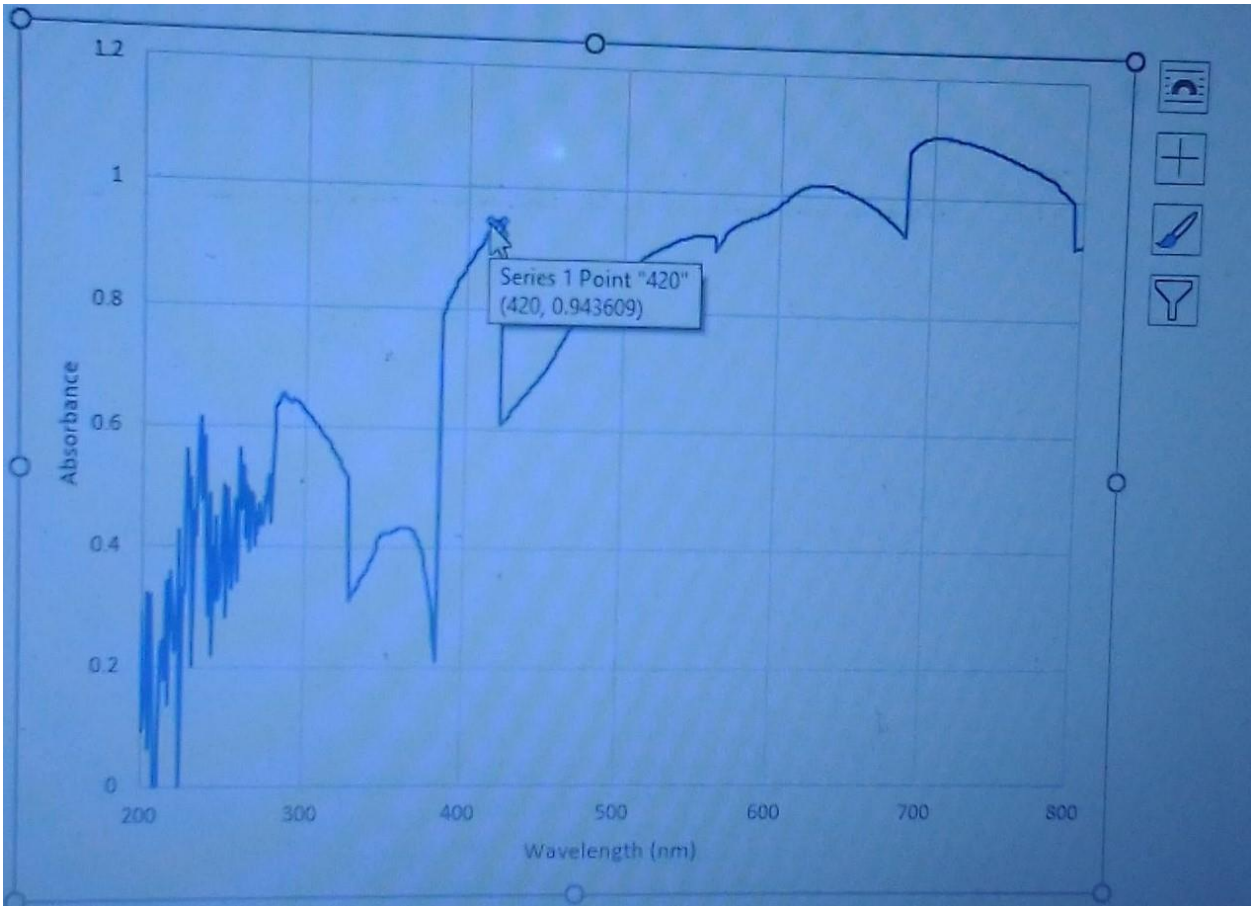

Figure 4.1.3: Spectrophotometric characterization of the nano extract of *Hibiscus sabdariffa*

4.1.4 Result of the 100nm resolution of Transmission Electron Microscopic analysis of the nano extract of *Hibiscus sabdariffa*

Figure 4.1.4 shows the 100 nm resolution of the microscopic study of the nano extract done using Transmission electron microscope. The microscopic study gave agglomerated spherical silver nanoparticles with average size of 65.9 nm.

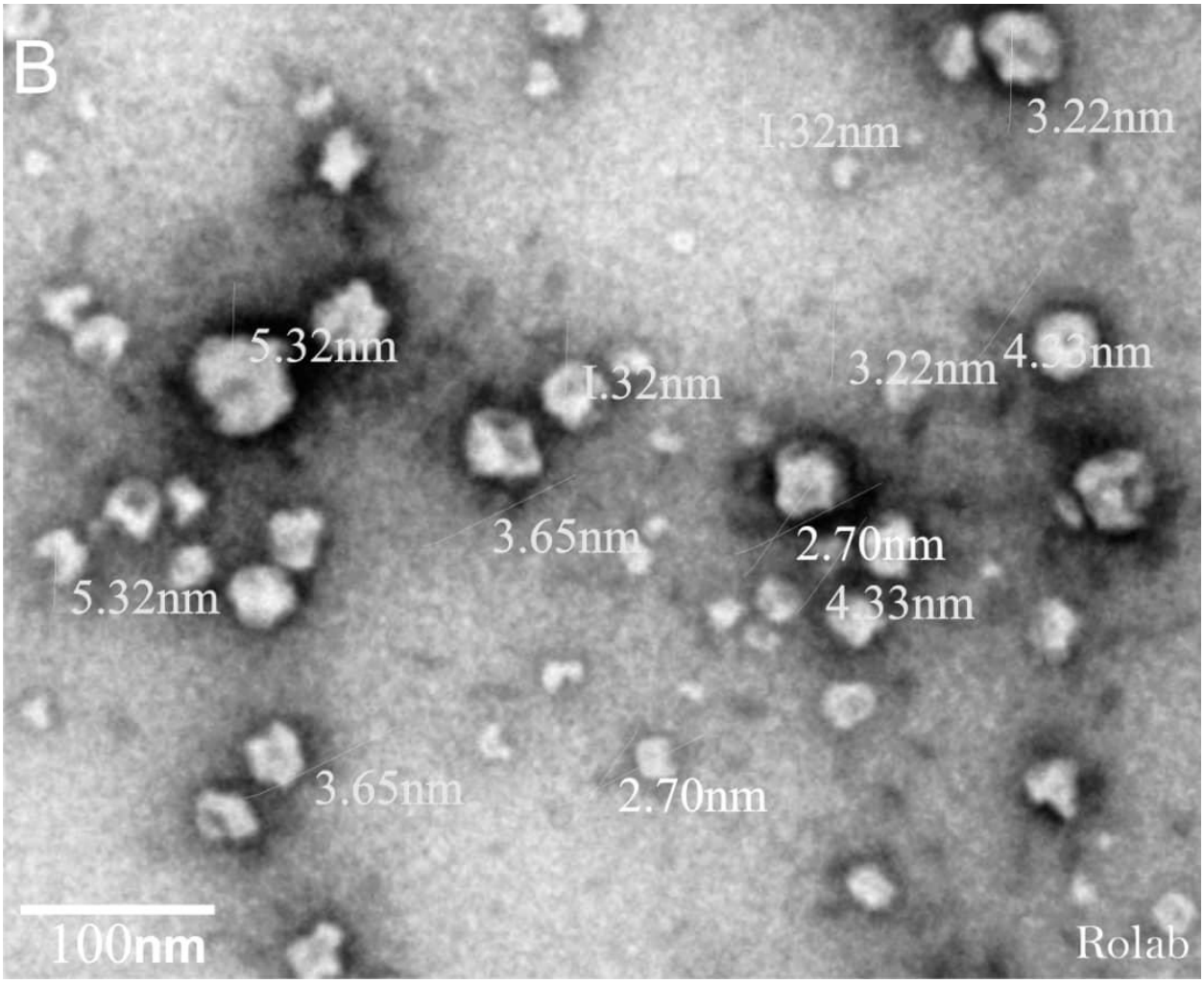

Figure 4.1.4: 100nm resolution of Transmission Electron Microscopic analysis of the nano extract of *Hibiscus sabdariffa*

4.1.5 Result of the150nm resolution of Transmission Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

Figure 4.1.5 shows the 150 nm resolution of the microscopic study of the nano extract done using Transmission electron microscope.

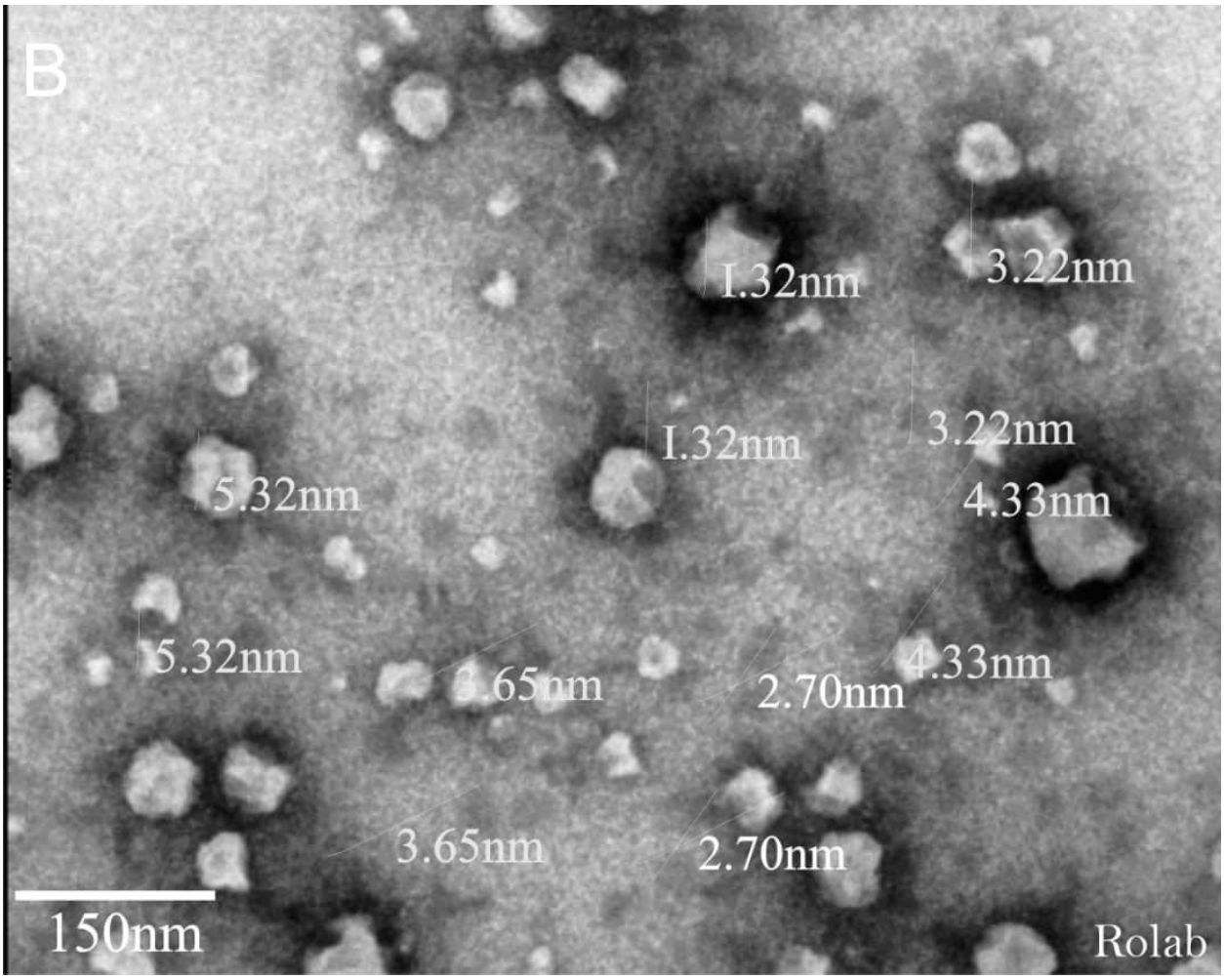

Figure 4.1.5: 150nm resolution of Transmission Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

4.1.6 Result of the 200 nm resolution of Transmission Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

Figure 4.1.6 shows the 200 nm resolution of the microscopic study of the nano extract done using Transmission electron microscope. The result showed agglomerated spherical silver nanoparticle with average size of 65.9 nm.

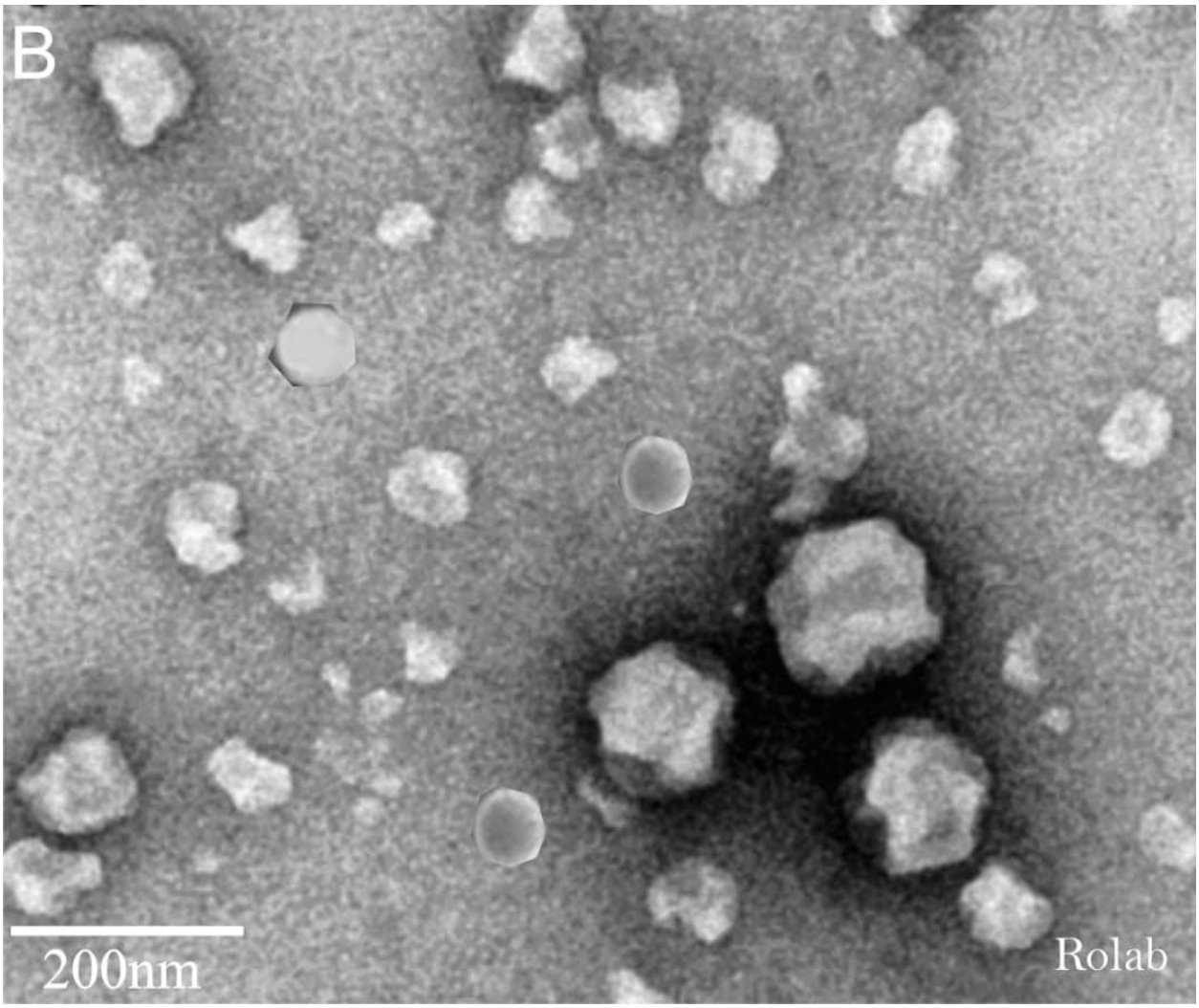

Figure 4.1.6: 200 nm resolution of Transmission Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

4.1.7 Result of the 100 µm Scanning Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

Figure 4.1.7 shows the 100 µm resolution of the Scanning electron microscopic result of the nano extract, which is meant to show the size and morphology of the nanoparticle but TEM gives a better and more specified size and shape.

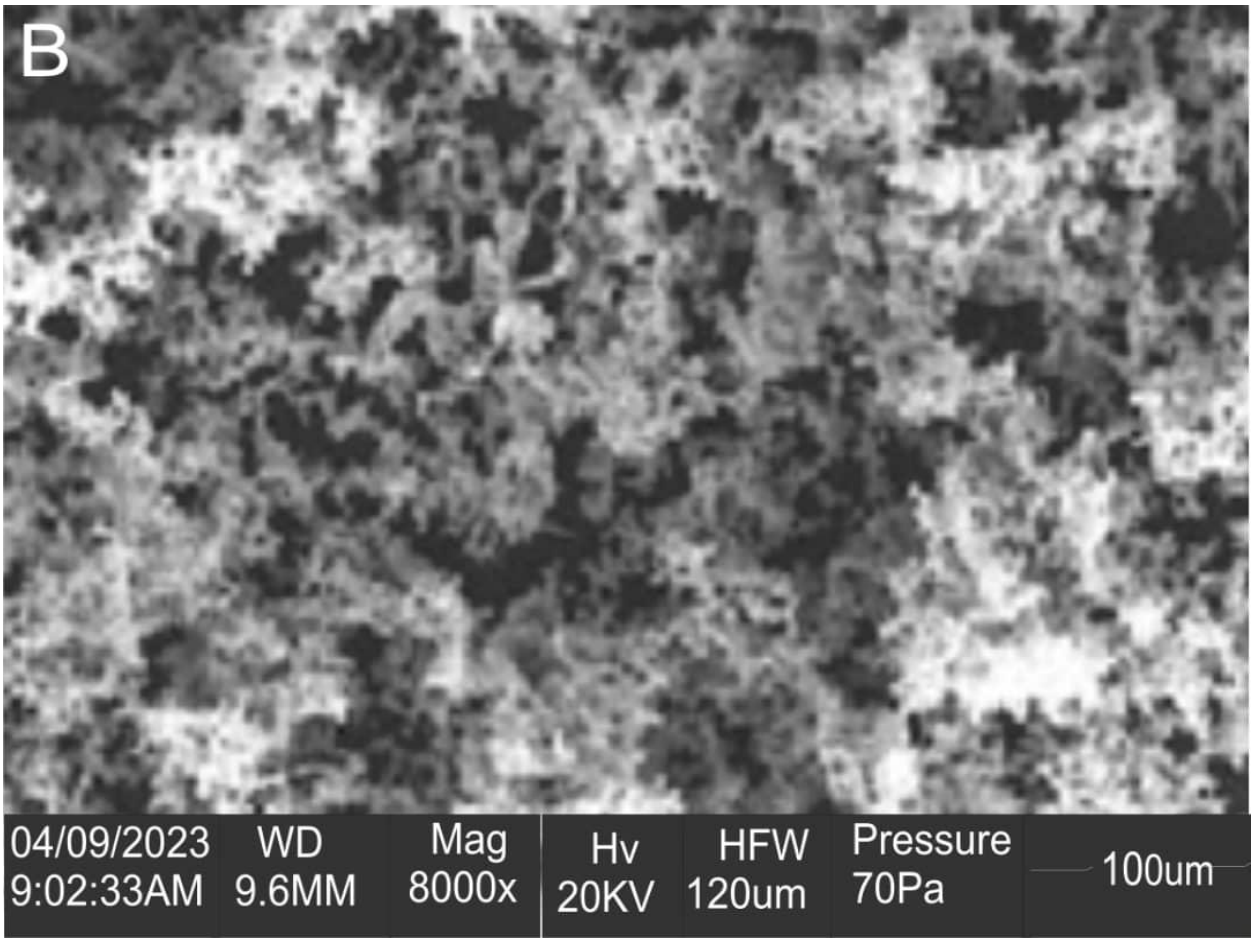

Figure 4.1.7: 100 µm Scanning Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

4.1.8 Result of the 20 µm Scanning Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

Figure 4.1.8 shows the 20 µm resolution of the Scanning electron microscopy of the nanoparticle.

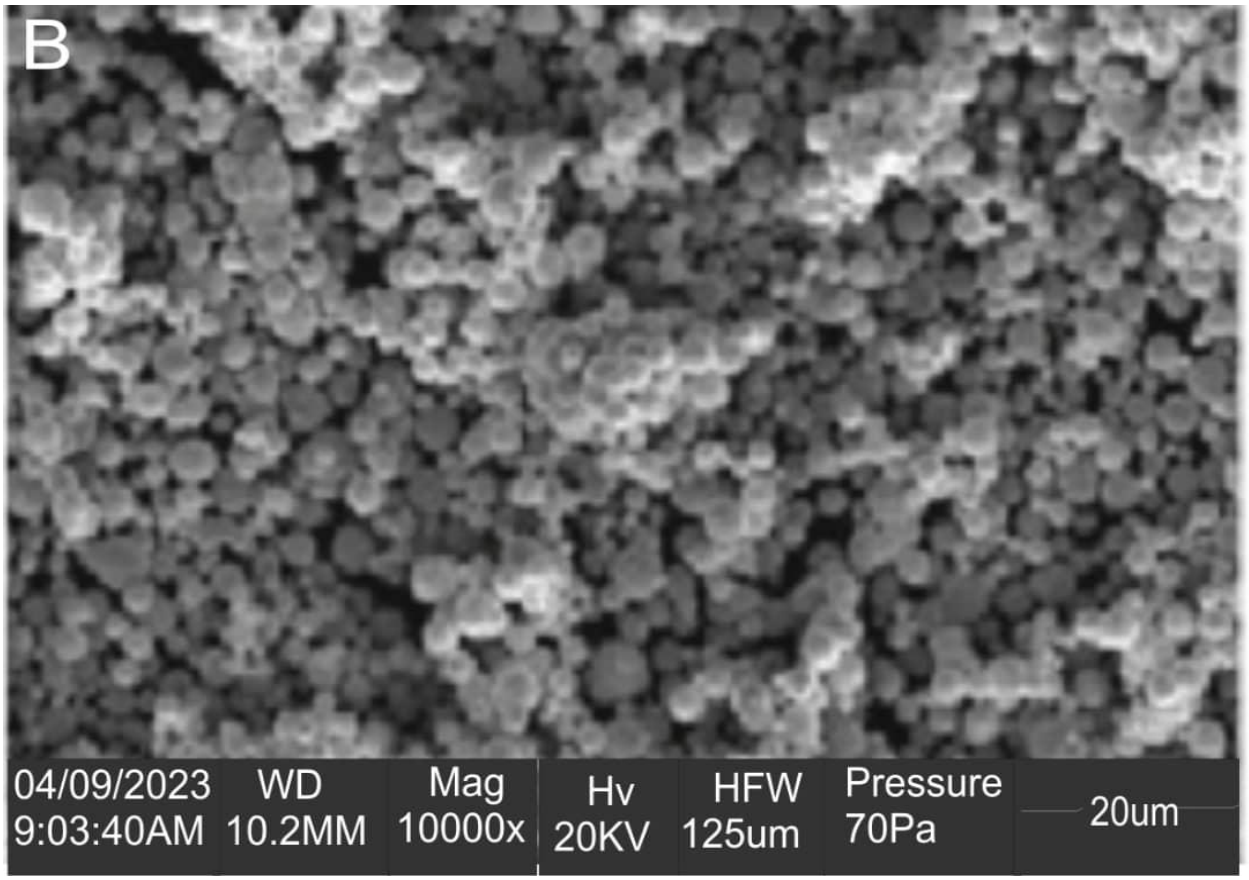

Figure 4.1.8: 20 µm Scanning Electron Microscopic analysis of *Hibiscus sabdariffa* nano extract

4.1.9 Energy Dispersive Spectroscopic Analysis (EDX) of *Hibiscus sabdariffa* nano extract

Figure 4.1.9 shows the Energy Dispersive Spectroscopic Analysis (EDX) of *Hibiscus sabdariffa* silver nanoparticle. The result revealed 79.52% Ag (silver) and 20.48% O (Oxygen). This shows the presence of elemental silver. The high percentage of silver indicates the presence of silver nanoparticle.

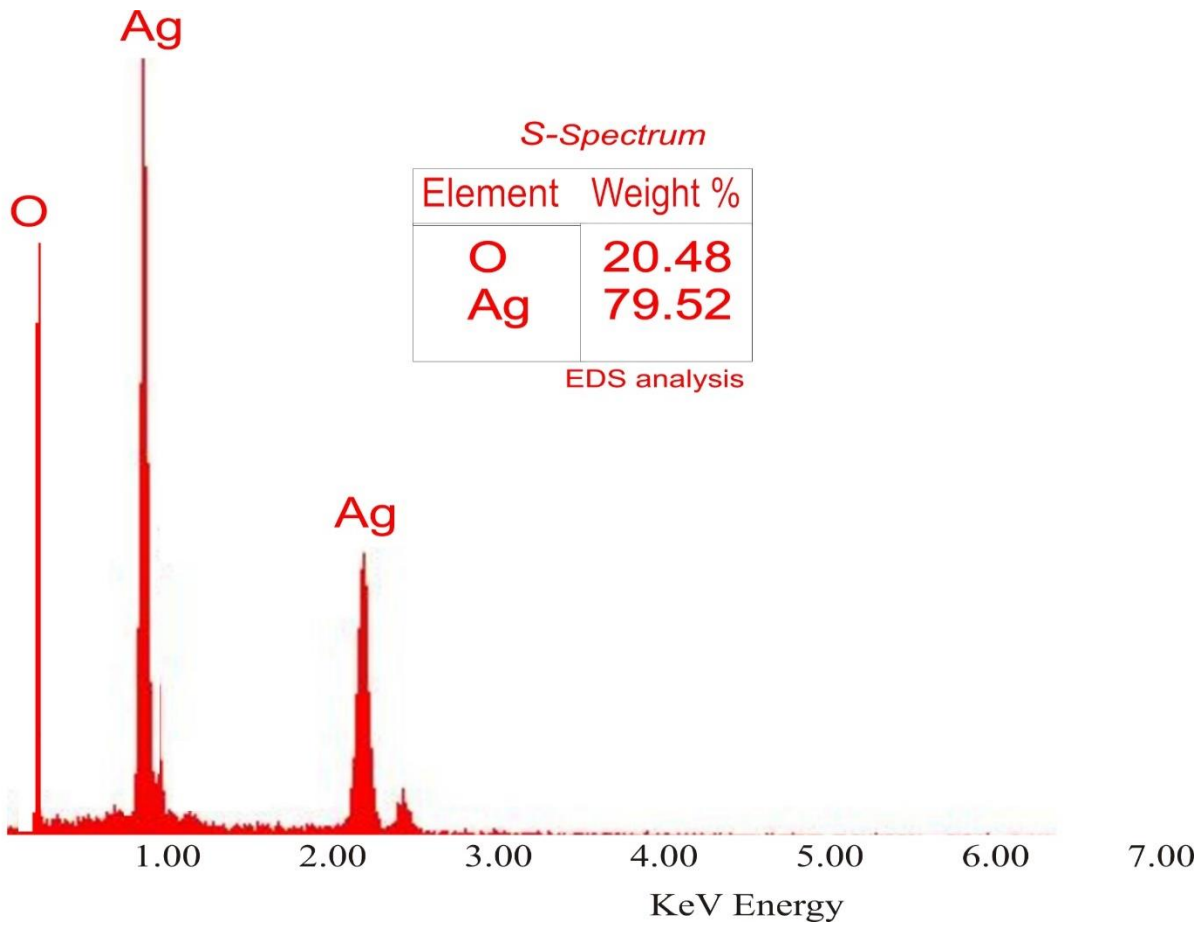

Figure 4.1.9: Energy Dispersive Spectroscopic Analysis (EDX) of *Hibiscus sabdariffa* nano extract

4.1.10 X-ray Diffraction analysis of *Hibiscus sabdariffa* nano extract

Figure 4.1.10 shows the X-ray Diffraction analysis of *Hibiscus sabdariffa* silver nanoparticle. The result showed that the nanoparticle exhibited a polycrystalline face cubic (FCC) structure with an average crystalline size of 65.9 nm. the nanoparticle showed a preferred orientation /texture as indicated by the relative peak intensities and the crystalline structure is relatively defect free with minimal lattice strain.

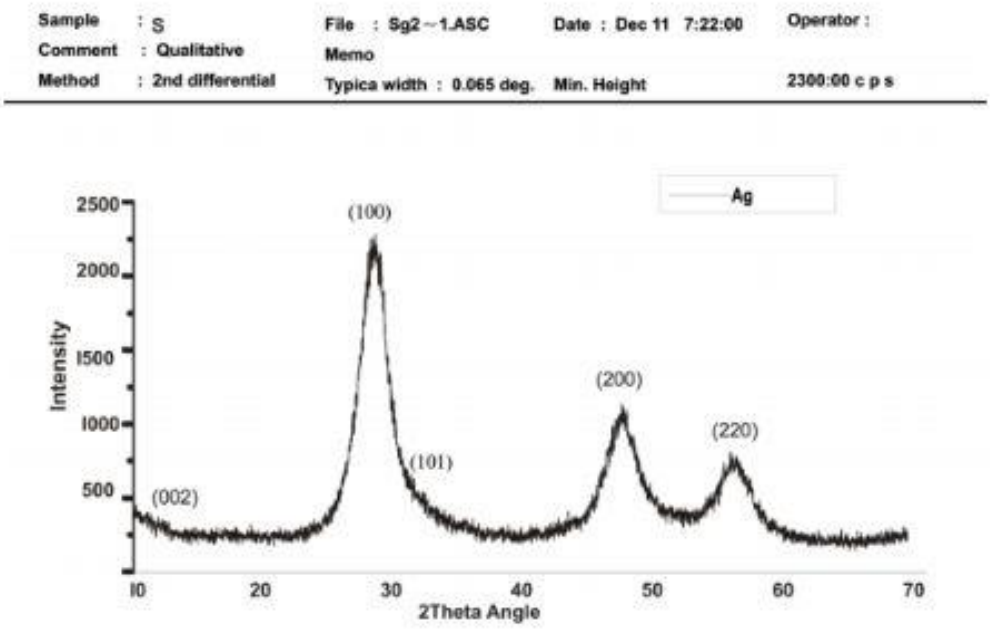

Figure 4.1.10: X-ray Diffraction analysis of *Hibiscus sabdariffa* nano extract

4.1.11 DPPH free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

Figure 4.1.11 shows the percentage inhibition (IC_{50}) curve of DPPH for the crude and nano extract of *Hibiscus sabdariffa* flower. The lower the IC_{50} the higher the scavenging ability of the extract. It shows the concentration of the extract that will be able to scavenge 50% of the radical. The crude extract recorded an IC_{50} of 0.42 mg/ml for DPPH radical while the nano recorded an IC_{50} of 6.2 mg/ml for DPPH radical.

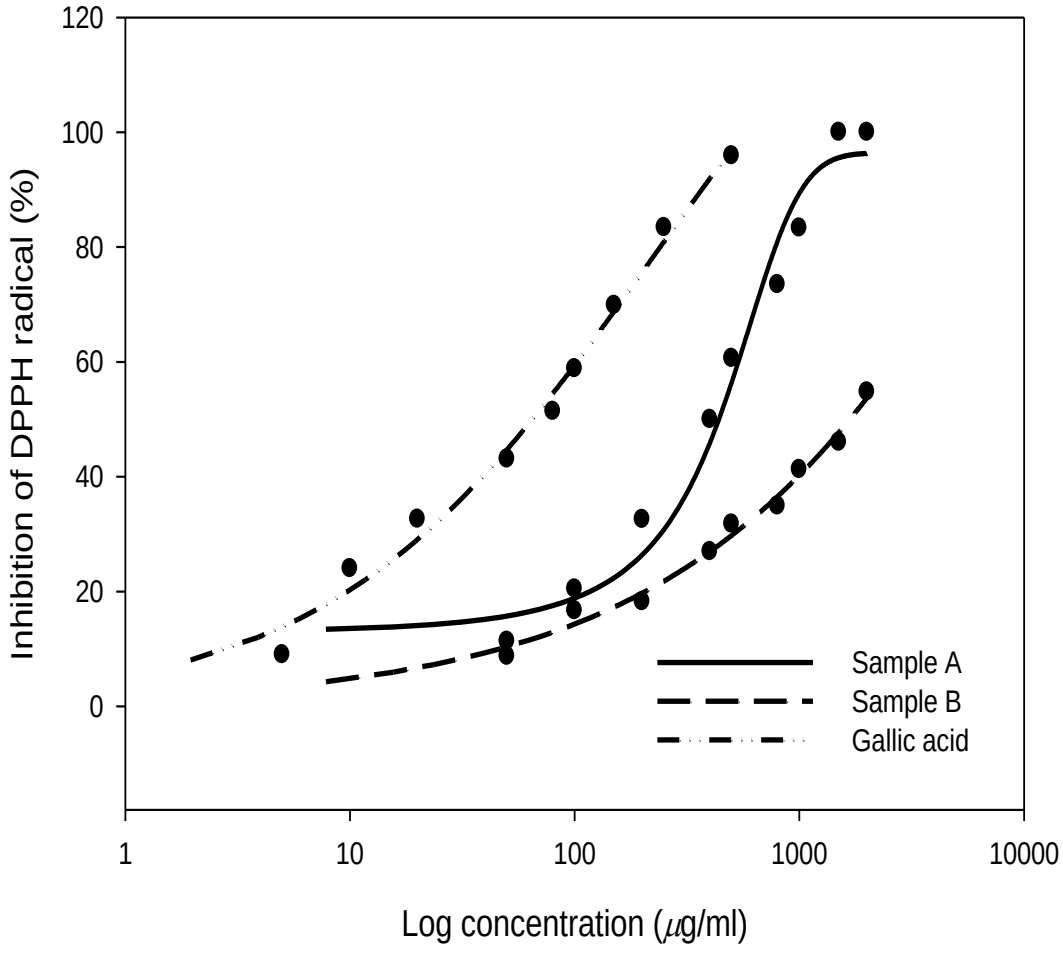

Fig.4.11: DPPH free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

4.1.12 Nitric oxide free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower

Figure 4.1.12 shows the percentage inhibition curve of Nitric oxide radical for the crude and nano extract of *Hibiscus sabdariffa* flower. The crude extract recorded an IC_{50} of 0.38 mg/ml while the nano recorded an IC_{50} of 0.72 mg/ml for nitric oxide radical.

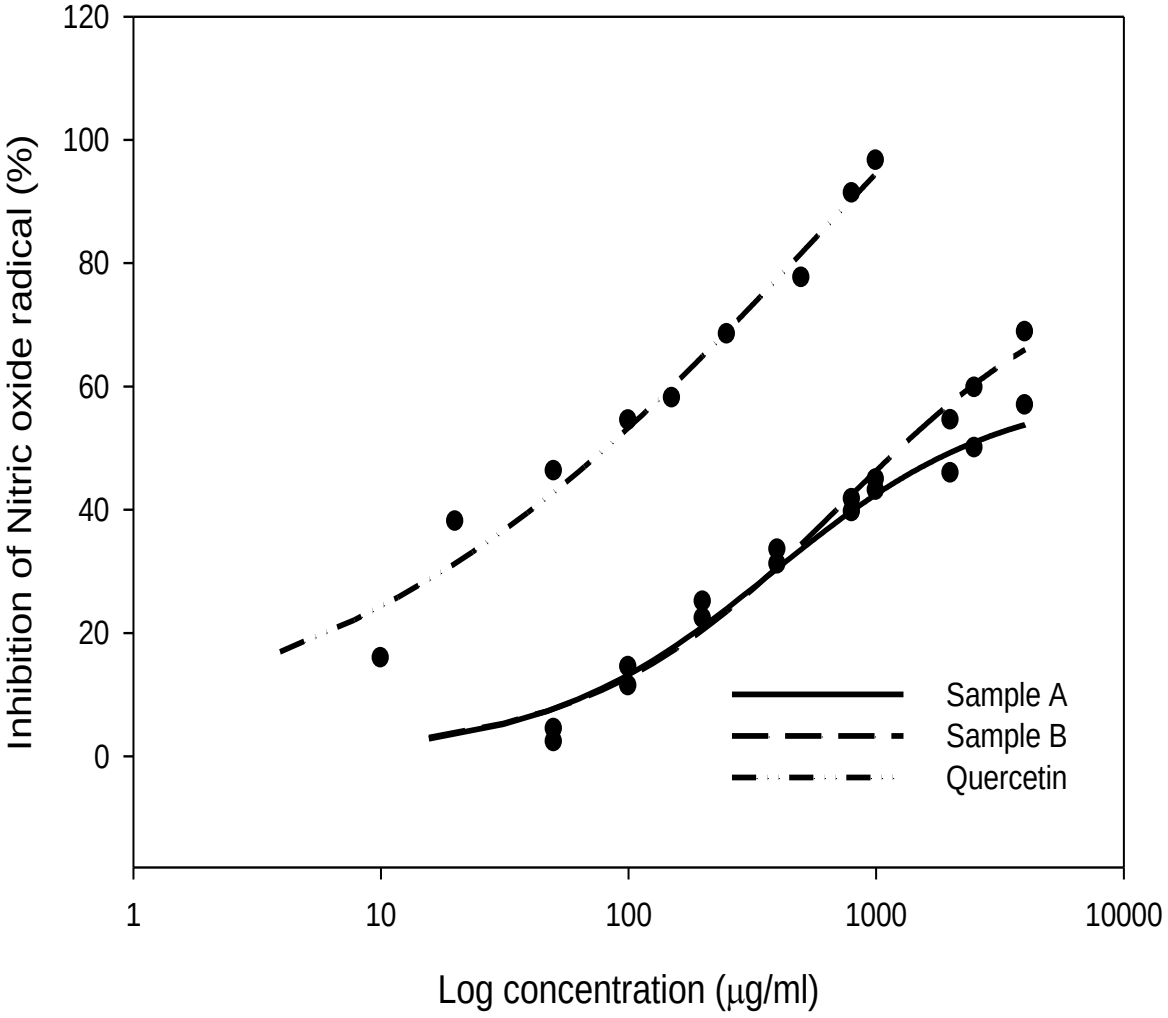

Figure 4.1.12: Nitric oxide free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

4.1.13 Hydroxyl radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

Figure 4.13 shows the percentage inhibition curve (IC_{50}) of hydroxyl radical for the crude and nano extract of *Hibiscus sabdariffa* flower. The crude recorded an IC_{50} of 0.33 mg/ml while the nano recorded an IC_{50} of 0.28 mg/ml for hydroxyl radical.

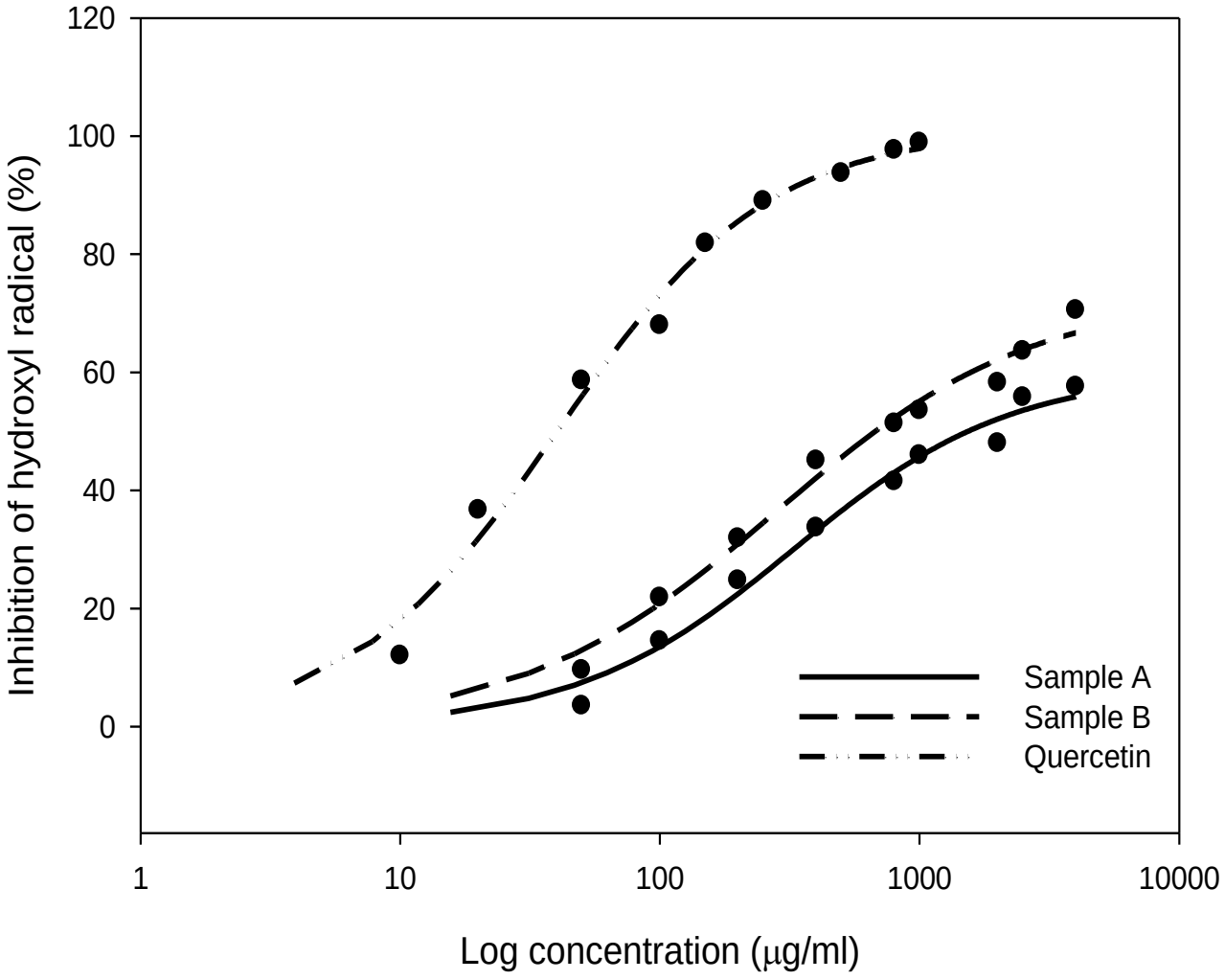

Figure 4.13: Hydroxyl radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

4.1.14 Superoxide free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

Figure 4.1.14 shows the percentage inhibition curve (IC_{50}) of superoxide radical for the crude and nano extract of *Hibiscus sabdariffa* flower. The crude recorded an IC_{50} of 0.67 mg/ml while the nano recorded an IC_{50} of 0.66 mg/ml for superoxide radical.

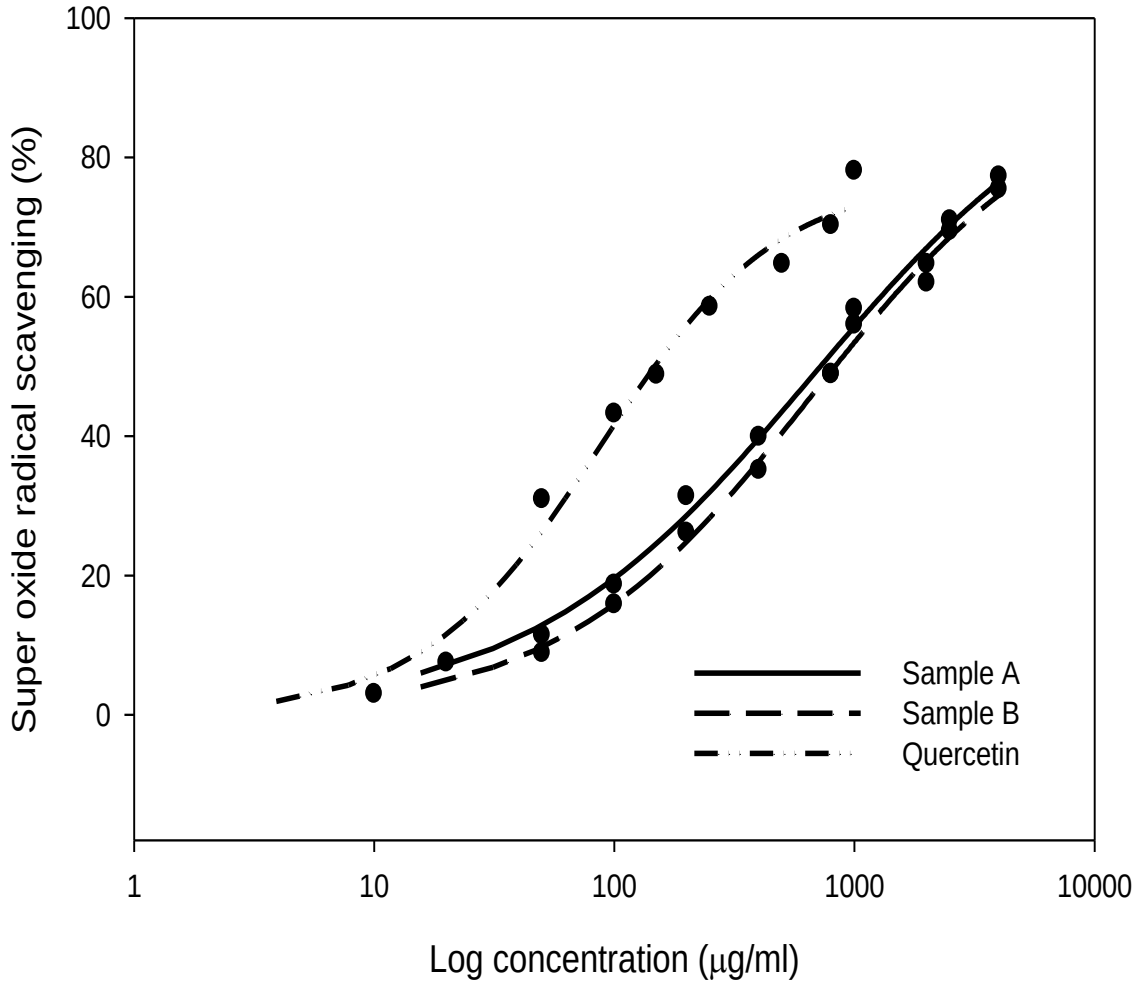

Figure 4.14: Superoxide free radical scavenging (%inhibition) effect of crude and nano extracts of *Hibiscus sabdariffa* flower.

Inhibitory concentration table for the crude and nano extract of *Hibiscus sabdariffa* flower

Table 4.1.3 shows the inhibitory concentration table for the crude and nano extract of *Hibiscus sabdariffa* flower. The unit was converted from µg/ml to mg/ml for ease of interpretation. The result shows the IC_{50} of the crude and nano extract for various radicals. The crude extract recorded an IC_{50} of 0.42 mg/ml for DPPH radical while the nano recorded an IC_{50} of 6.2 mg/ml for DPPH radical. The crude extract recorded an IC_{50} of 0.38 mg/ml while the nano recorded an IC_{50} of 0.72 mg/ml for nitric oxide radical. The crude recorded an IC_{50} of 0.33 mg/ml while the nano recorded an IC_{50} of 0.28 mg/ml for hydroxyl radical. The crude recorded an IC_{50} of 0.67 mg/ml while the nano recorded an IC_{50} of 0.66 mg/ml for superoxide radical.

Table 4.1.3: Inhibitory concentration table for the crude and nano extract of *Hibiscus sabdariffa* flower

Parameters	Sample	Threshold Inhibitory concentration (IC_{50}) (mg/ml)	Model Equation	R^2
DPPH radical Scavenging	A (C.E)	0.422 ± 13.92^{a}	**Sigmoid, 3 Parameter**	0.967
	B (N.E)	6.160 ± 49.28^{b}	**Logistic, 3 Parameter**	0.992
	Gallic acid	0.214 ± 1.71^{c}	**Logistic, 3 Parameter**	0.992
Nitric oxide radical scavenging	A (C.E)	0.382 ± 4.20^{d}	**Logistic, 3 Parameter**	0.989
	B (N.E)	0.721 ± 10.81^{e}	**Logistic, 3 Parameter**	0.985
	Quercetin	0.609 ± 10.98^{f}	**Logistic, 3 Parameter**	0.982
Hydroxyl radical scavenging	A (C.E)	0.327 ± 3.92^{g}	**Logistic, 3 Parameter**	0.988
	B (N.E)	0.282 ± 3.10^{h}	**Logistic, 3 Parameter**	0.989
	Quercetin	0.041 ± 0.78^{i}	**Logistic, 3 Parameter**	0.981
Super oxide radical scavenging	A (C.E)	0.669 ± 3.34^{J}	**Logistic, 3 Parameter**	0.995
	B (N.E)	0.664 ± 2.65^{J}	**Logistic, 3 Parameter**	0.996
	Quercetin	0.088 ± 1.14^{k}	**Logistic, 3 Parameter**	0.987

Values are mean ± standard deviation of 3 determinations. Mean values with different superscript are significantly different ($p<0.05$)

4.1.15 Total Antioxidant Capacity of the Crude and Nano Extract of *Hibiscus sabdariffa* flower.

Figure 4.1.15 shows the result of the total antioxidant capacity of the crude and nano extract of *hibiscus sabdariffa* flower. The result showed a higher antioxidant capacity of the nano extract compared to the crude extract.

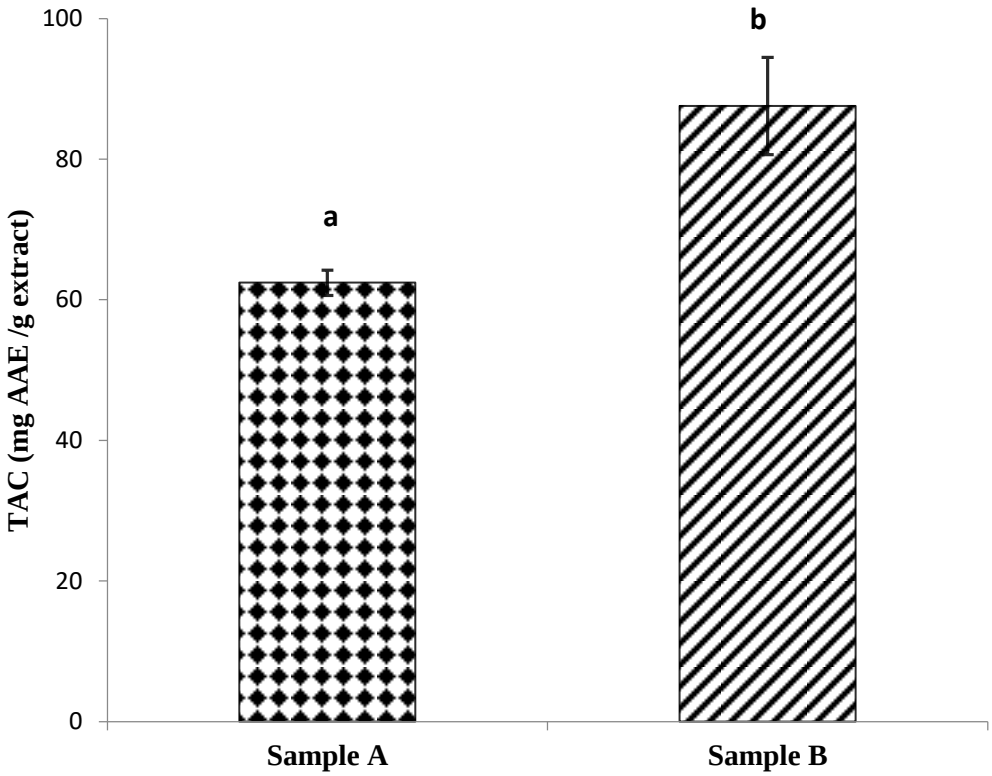

Figure 4.1.15: Total Antioxidant Capacity of the Crude and Nano Extract of *Hibiscus sabdariffa* flower.

Results of the antimicrobial analysis of the crude and nano extract of *Hibiscus Sabdariffa* using spectrophotometric assay:

This table shows the antimicrobial activity of *Hibiscus Sabdariffa* crude and nano extracts against the test organisms: *Staphylococcus aureus, Escherichia coli (E. coli*) and *Salmonella typhi.* The crude extract inhibited *staphylococcus aureus* and *E. coli* only at the highest concentration of 500 mg/ml with a zone of inhibition of 17.5mm and 19mm respectively, *Salmonella. typhi* showed a dose dependent response while the nano extract exhibited higher antimicrobial activity than the crude extract.

Table 4.1.4: Sensitivity of *Hibiscus sabdariffa* Crude and Nano Extract on Test Organisms in Diameter (mm)

Test Isolates	*Sample Code*	*500 mg/ml*	*250 mg/ml*	*125 mg/ml*	*62.5 mg/ml*	*CIP*
Staphylococcus aureus	A	17.5	0	0	0	45
Escherichia Coli	A	19	0	0	0	41
Salmonella Typhi	A	22.5	19	17.5	0	45
Staphylococcus aureus	B	27.5	32.5	22.5	25	42.5
Escherichia Coli	B	26	22.5	17.5	17.5	37.5
Salmonella Typhi	B	32.5	27.5	22.5	20	45

Ciprofloxacin. Sample A: Crude extract ; Sample B: Nano extract of Hs

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4.1.16 Sensitivity of *Hibiscus sabdariffa* Crude and Nano Extract on Test Organisms in Diameter (mm)

Figure 4.1.16 shows the Inhibitory Concentration Curves for *Hibiscus Sabdariffa* crude extract against *E. coli, S. aureus and Salmonella* species.

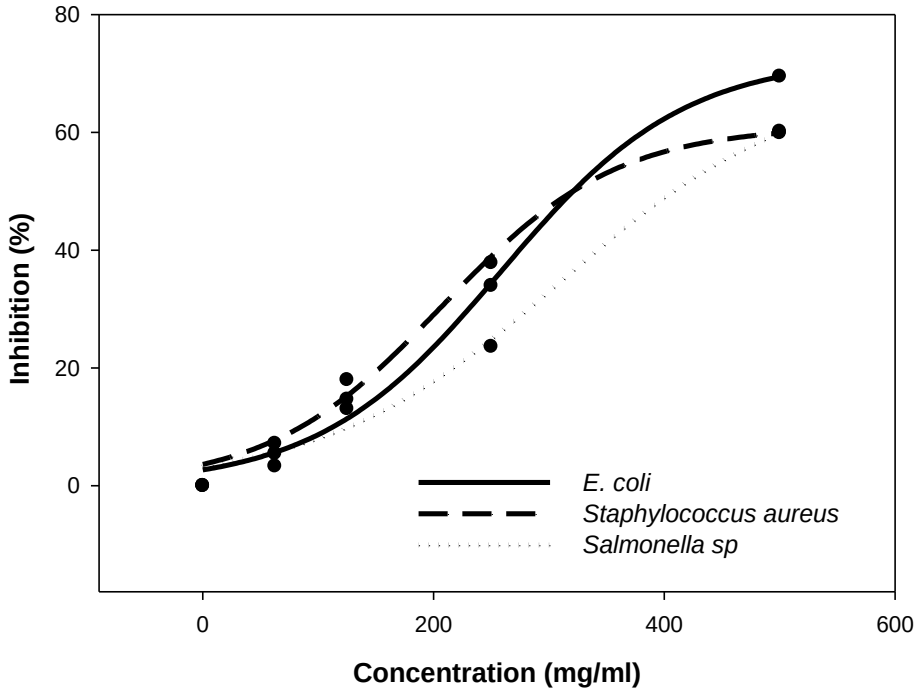

Figure 4.1.16: Inhibitory Concentration Curves for *Hibiscus Sabdariffa* crude extract against *E. coli, S. aureus and Salmonella* species.

Figure 4.1.17 shows Inhibitory Concentration Curve for *Hibiscus Sabdariffa* Nano extract against *E.coli, S.aureus and Salmonella species*.

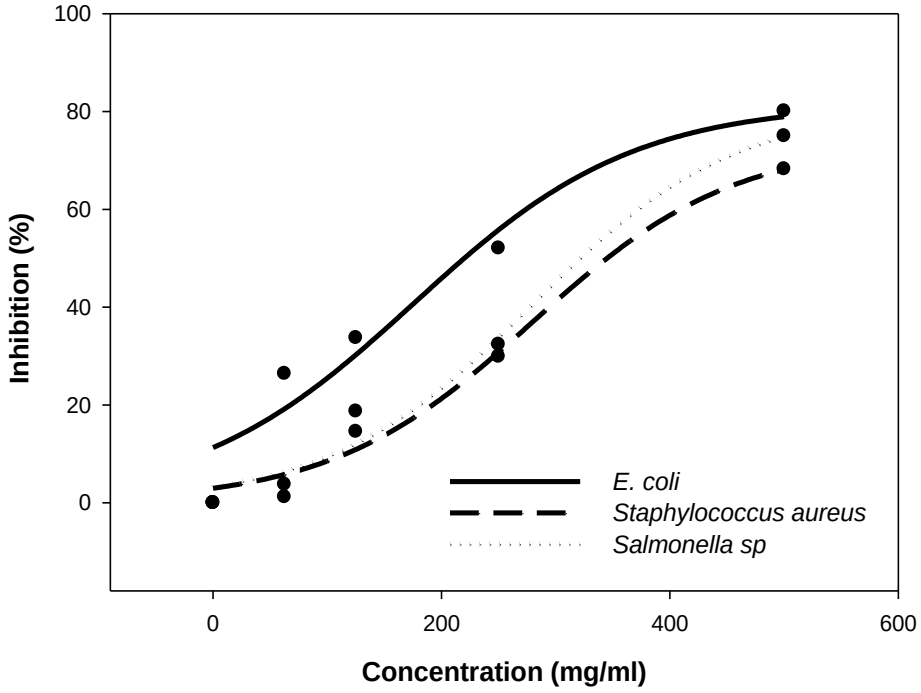

Figure 4.1.17: Inhibitory Concentration Curve for *Hibiscus Sabdariffa* Nano extract against *E.coli, S.aureus and Salmonella specie*

Inhibitory concentration table of *Hibiscus sabdariffa* crude and nano extract against bacteria isolates.

Table 4.1.5 shows the Threshold Inhibitory Concentration (IC_{50}) (mg/ml) table for the crude and nano extract. The lower the IC_{50} the higher the antimicrobial potency. The nano extract recorded an IC_{50} of 259 mg for *E.coli* while the nano recorded 176 mg. The crude recorded an IC_{50} of 208 mg for *Staph. aureus* while the nano extract recorded 279 mg. The crude recorded an IC_{50} of 310 mg for *Salmonella. typhi* while the nano recorded 279 mg. This shows an Improved antimicrobial property for the nano extract especially for *E. coli* and *Salmonella. typhi*.

Table 4.1.5: Inhibitory concentration table of *Hibiscus sabdariffa* crude and nano extract against bacteria isolates.

Threshold Inhibitory Concentration (IC_{50}) (mg/ml) table

Isolates	*A*	R^2	*B*	R^2
E. coli	258.51± 14.60	0.9968	176.04±15.30	0.9419
Staphylococcus aureus	207.65± 18.61	0.9908	279.31±13.92	0.9912
Salmonella sp	309.91±17.75	0.9823	279.34±25.00	0.9775

Sample A: crude extract; Sample B; Nano extract

4.2 DISCUSSION

The GC-MS result of the crude extract of *Hibiscus Sabdariffa* leaf shows a total of fifteen bio compounds which has 33.82 % of 4-amino-1,2 naphtoquinone occurring highest while hexadecenoic acid methyl ester occurs least at 0.36%, with their molecular weights ranging from 173 – 308 and their percentage composition ranging from 0.36 – 33.82%. Majority of these biological compounds are quinones, fatty acid methyl esters (FAME), glycosides, benzamides and so on which are responsible for the plants antioxidant, anti-inflammatory, anticancer, antimicrobial activities and cardiovascular health benefits. This finding is in line with the findings of (Kumar and Sheba, 2019), who also observed majority of these phytochemicals in *Hibiscus sabdariffa.*

The result of the GC-MS of the nano extract recorded fifteen (15) organic compounds with Cis-Vaccenic acid occurring highest at 46.37% while N-2-carbomethoxyvinyl methylamine occurs lowest at 0.93%. Their molecular weights ranges from 84-356 while their composition ranges from 0.93-46.37%. These organic compounds contain polymers, lipids and other carbon-based molecules which are responsible for the nanoparticle anti-inflammatory, antioxidant, antimicrobial effects and its cardiovascular health support and nutritional value.

The spectrophotometric result of the nano extract showed maximum light absorption at a wavelength of 420 nm. This indicates that the silver nanoparticle exhibits a strong surface plasmon resonance (SPR) at 420 nm, which could be as a result of the collective oscillations of electrons on the surface of the nanoparticles which are excited by the incident light. The initial roughness of the curve could be as a result of solvent or impurity effects which affected the lower wavelength region while the smoothing out of the curve at higher wavelengths suggests

that the nanoparticle absorption dominates. This result is in line with the spectrophotometric result obtained by Khwatr et al., (2020).

The microscopic studies gave agglomerated spherical silver nanoparticle with average size of 65.9 nm. This indicates that the silver nanoparticles are in the nanoparticle range, which is consistent with the expected size range for nanoparticles. It also suggests that the nanoparticles are relatively uniform in size, which is desirable for many applications. This size of the nanoparticles can also influence their properties such as the optical properties – exhibiting strong surface plasmon resonance (SPR) absorption, which is consistent with the UV-VIS result (420 nm). It can influence catalytic activity because nanoparticle of this size can have high surface areas, making them suitable for catalytic applications. It can also influence its biological interactions because nanoparticle of this size can interact with cells and biomolecules, making them suitable for biomedical applications.

The EDX revealed 79.52% Ag (silver) and 20.48% O (oxygen). This shows the presence of elemental silver. The high percentage of silver confirms the presence of silver nanoparticles. The presence of oxygen suggests that the silver nanoparticles are oxidized, possibly due to surface oxidation, silver oxide formation, residual solvent or moisture, possible silver oxide phases, surface modification or less likely contamination from environment or sample handling.

The XRD result shows that the silver nanoparticle exhibits a polycrystalline face centered cubic (FCC) structure with an average crystallite size of 65.9 nm. The nanoparticles show a preferred orientation or texture as indicated by the relative peak intensities and the crystalline structure is relatively defect free with minimal lattice strain. This XRD result provided a complementary information with that of TEM analysis result.

The crude extract recorded an IC_{50} of 0.42 mg/ml for DPPH radical while the nano extract recorded an IC_{50} of 6.2 mg/ml for DPPH. The crude extract has higher DPPH radical scavenging activity than the nano extract.

The crude extract recorded an IC_{50} of 0.38 mg/ml for Nitric oxide radical while the nano extract recorded an IC_{50} of 0.72 mg/ml for nitric oxide radical. The crude extract has higher nitric oxide scavenging activity than the nano extract.

The result of the crude extract for hydroxyl radical scavenging activity recorded an IC_{50} of 0.33 mg/ml for hydroxyl radical while that of the nano extract recorded an IC_{50} of 0.28 mg/ml for hydroxyl radical suggesting that the nano extract has a slightly enhanced hydroxyl radical scavenging activity which might be due to its smaller particle size and increased surface area.

Result of the crude extract for superoxide radical scavenging activity recorded an IC_{50} of 0.67 mg/ml while that of the nano extract recorded an IC_{50} of 0.66 mg/ml suggesting that the nano extract has similar superoxide radical scavenging activity to the crude extract indicting that the nanosization process did not significantly impact this aspect of antioxidant activity.

The result of the total antioxidant capacity showed a higher total antioxidant capacity of the nano extract compared to the crude extract, this could be as a result of: **Different antioxidant mechanisms** -The nano extract might have a higher capacity to donate electrons or higher atoms, contributing to its higher total antioxidant capacity despite having lower DPPH and nitric oxide scavenging ability. It might be as a result of synergistic effects: The nano extract might contain a combination of bioactive compounds that work synergistically to enhance total antioxidant capacity even if individual compounds have lower DPPH and nitric oxide scavenging activity. It might be as a result of increased bioavailabilty: the nano extract smaller particle size might enhance its bioavailability, allowing it to interact more effectively with other antioxidants or

cellular components, contributing to its higher total antioxidant capacity. It might also be as a result of different antioxidant assays: The total antioxidant capacity assay might be more sensitive to certain types of antioxidants or mechanisms whereas DPPH and nitric oxide assays are more specific to free radical scavenging. These findings suggest that the nano extract has a unique antioxidant profile compared to the crude extract, with enhanced total antioxidant capacity and hydroxyl radical scavenging activity but lower DPPH and nitric oxide radical scavenging activity. The radical scavenging properties of this *Hibiscus sabdariffa* silver nanoparticles are attributed to the presence of phenolic compounds, flavonoids and saponins which can donate electrons to neutralize free radicals. These findings are somehow related to the findings of Alaa, (2012) that *Hibiscus sabdariffa* exhibited a high antioxidant property and the findings of Mohammad, Mehrnaz & Amir, (2018) that silver nanoparticle also exhibited a high antioxidant activity.

Antimicrobial activity of *Hibiscus Sabdariffa* crude and nano extracts against three microorganisms: *Staphylococcus aureus, Escherichia coli (E. coli*) and *Salmonella typhi.* showed the following results:

For the crude extract: *Staphyloccocus aureus* inhibited only at the highest concentration (500 mg/ml) with a Zone of inhibition (ZOI) of 17.5mm. *E.coli* inhibited only at the highest concentration (500 mg/ml) with a ZOI of 19mm while *Salmonella typhi* showed a dose-dependent response, with increasing ZOI at higher concentrations (22.5mm at 500 mg/ml, 19mm at 250 mg/ml and 17.5mm at 125 mg/ml).

For *Staphylococcus aureus* the nano extract: showed increased antimicrobial activity at all concentrations with a maximum ZOI of 27.5mm at 500 mg/ml. For *E.coli*, the nano extract also

showed increased antimicrobial activity at all concentrations, with a maximum ZOI of 32.5mm at 250 mg/ml. with these results, it shows that:

The nano extract exhibited higher antimicrobial activity than the crude extract indicating improved bioavailability and efficacy. This somehow corresponded with the work of Raghad et al, (2020) whose work showed that the nano extract of *Hibiscus Sabdariffa* showed increased antimicrobial activity against the crude extract.

Salmonella typhi is the most susceptible to both extracts, followed by *E.coli* and then *Staphylococcus aureus*.

The crude extract shows limited antimicrobial activity, only inhibiting growth at the highest concentration (500 mg/ml) for *Staphylococcus aureus* and *E.coli.*

The nano extract demonstrated a dose-dependent response, with increasing antimicrobial activity at higher concentrations.

The overall results suggest that *Hibiscus Sabdariffa* nano extract has potential antimicrobial properties, particularly against Salmonella typhi and warrants further investigation for therapeutic applications.

The IC_{50} (half maximal inhibitory concentration) results show the concentration of *Hibiscus Sabdariffa* crude and nano extracts required to inhibit the growth of *E. coli, Staphylococcus aureus* and *Salmonella typhi* by 50%. Lower IC50 values indicate greater antimicrobial potency. From the result above, it showed that the nano extract is more potent against E. coli with a lower IC_{50} value.

The crude extract was more potent against *Staph. aureus* with a lower IC_{50} value despite the expectation that the nano should be more potent. This could be as a result of various reasons such as: Particle size and distribution: The nano extracts particle size and distribution might not be optimal for interacting with *staph. aureus* leading to reduced potency.

Surface chemistry: The surface chemistry of the nano extract might be less favourable for interacting with *Staph aureus* thus reducing its effectiveness.

Bacterial strain variability: The specific strain of s*taph. aureus* used in the test might be less susceptible to the nano extract.

Test conditions: The test conditions might not be optimal for the nano extract's activity against *staph. aureus*.

The nano extract is slightly more potent against *Salmonella typhi*, with a lower IC_{50} value. So in all, the nano extract exhibited improved antimicrobial potency against E. coli and Salmonella typhi compared to the crude extract

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSSION

Hibiscus sabdariffa silver nanoparticle has demonstrated the superior free radical scavenging and antimicrobial properties over the crude extract. The nano extract exhibited enhanced invitro antioxidant activity, it has a unique antioxidant profile compared to the crude extract, with enhanced total antioxidant capacity and hydroxyl radical scavenging activity but lower DPPH and nitric oxide radical scavenging activity, effectively neutralizing free radicals and showed improved antimicrobial efficacy against various pathogens especially. *E. coli* and *Salmonella typhi*.

These findings suggest that the Nano extract has potential applications in the development of natural based products for pharmaceutical, cosmetics and food industries.

5.2 Recommendations for further studies

Based on the results, we recommend further investigation into the following areas:

1. **Scale up production**: To optimize the synthesis process to produce larger quantities of *Hibiscus Sabdariffa* silver nanoparticles for industrial applications.
2. **Toxicity studies**: To conduct comprehensive toxicity assessments to ensure the safety of the nano extract for human consumption and topical use.
3. **Formulation development**: To explore the incorporation of the nano extract into various product formulations such as creams, lotions and capsules to enhance their antioxidant and antimicrobial properties.

4. **Clinical trials**: To conduct clinical trials to evaluate the efficacy of the nano extract in preventing or treating diseases related to oxidative stress and microbial infection.

Contribution to knowledge

We found out that:

1. *Hibiscus sabdariffa* silver nanoparticle has an enhanced hydroxyl radical scavenging ability and a higher total antioxidant capacity against the crude extract.
2. The nano extract of *Hibiscus sabdariffa* showed a dose dependent response, inhibiting microorganisms at various concentrations especially for *E. coli* and *Salmonella. typhi.*
3. The nano extract of *Hibiscus sabdariffa* were able to inhibit 50% of the organisms at lower concentrations especially for *E. coli* and *Salmonella. typhi.*
4. *Hibiscus sabdariffa silver* nanoparticle can be engineered for targeted delivery to specific cells or tissues, similar to liposomes and exosomes but with enhanced precision and efficacy.

   With the above, it could be seen that *Hibiscus sabdariffa* silver nanoparticle has a unique antioxidant profile and an improved antimicrobial potency especially for *E.coli* and *Salmonella. typhi* against the crude extract hence could serve as a potential antioxidant and antimicrobial agent that could used in the development of natural based products for pharmaceuticals, cosmetics and food industries.

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Appendix 1

Figure 2.9: Picture of *Hibiscus Sabdariffa* Leaf and Flower

Appendix 2

Figure 3.1: Nano extract preparation

Appendix 3

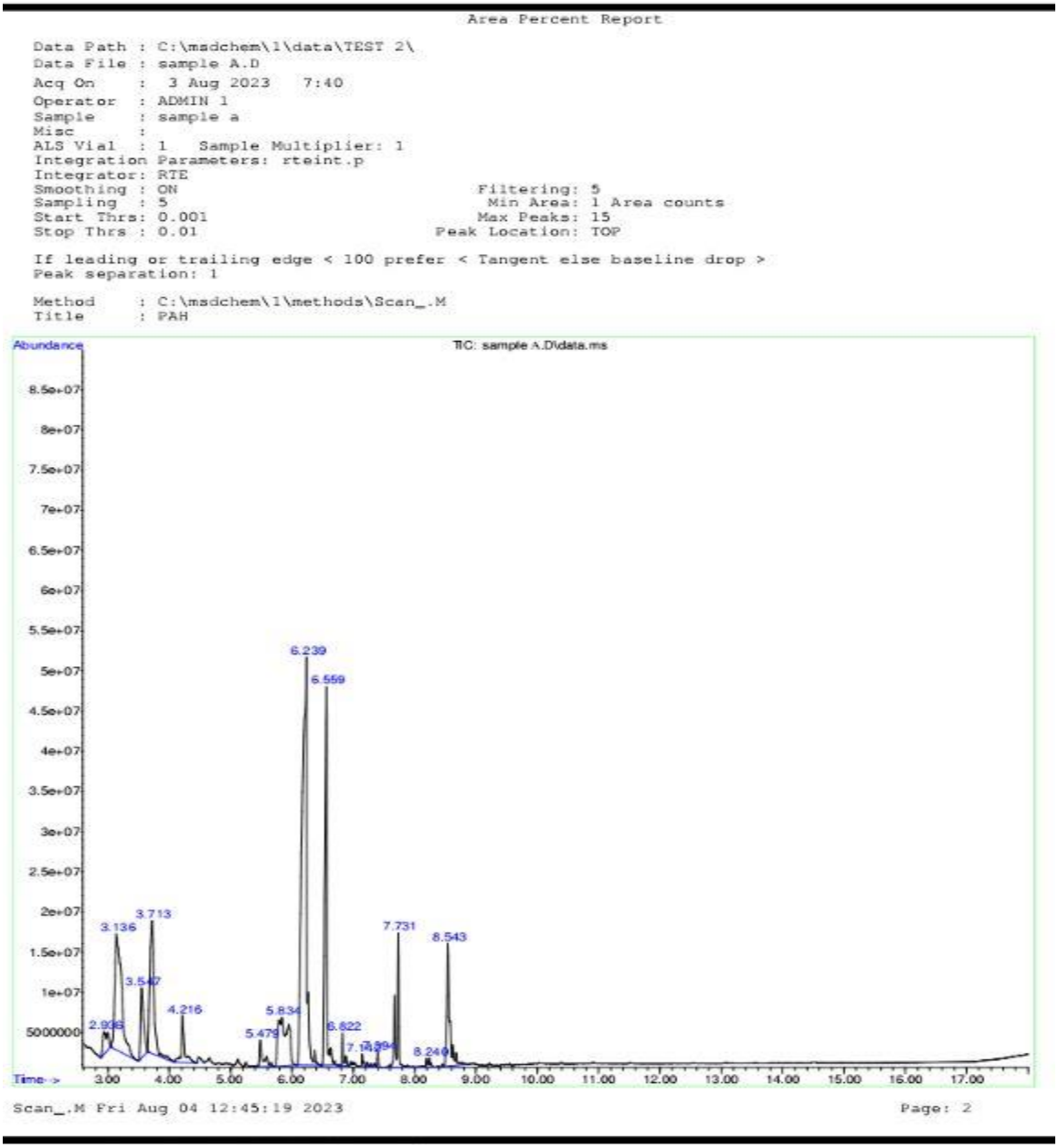


Figure 4.1.1: SAMPLE A (crude extract of Hibiscus sabdariffa) CHROMATOGRAPH

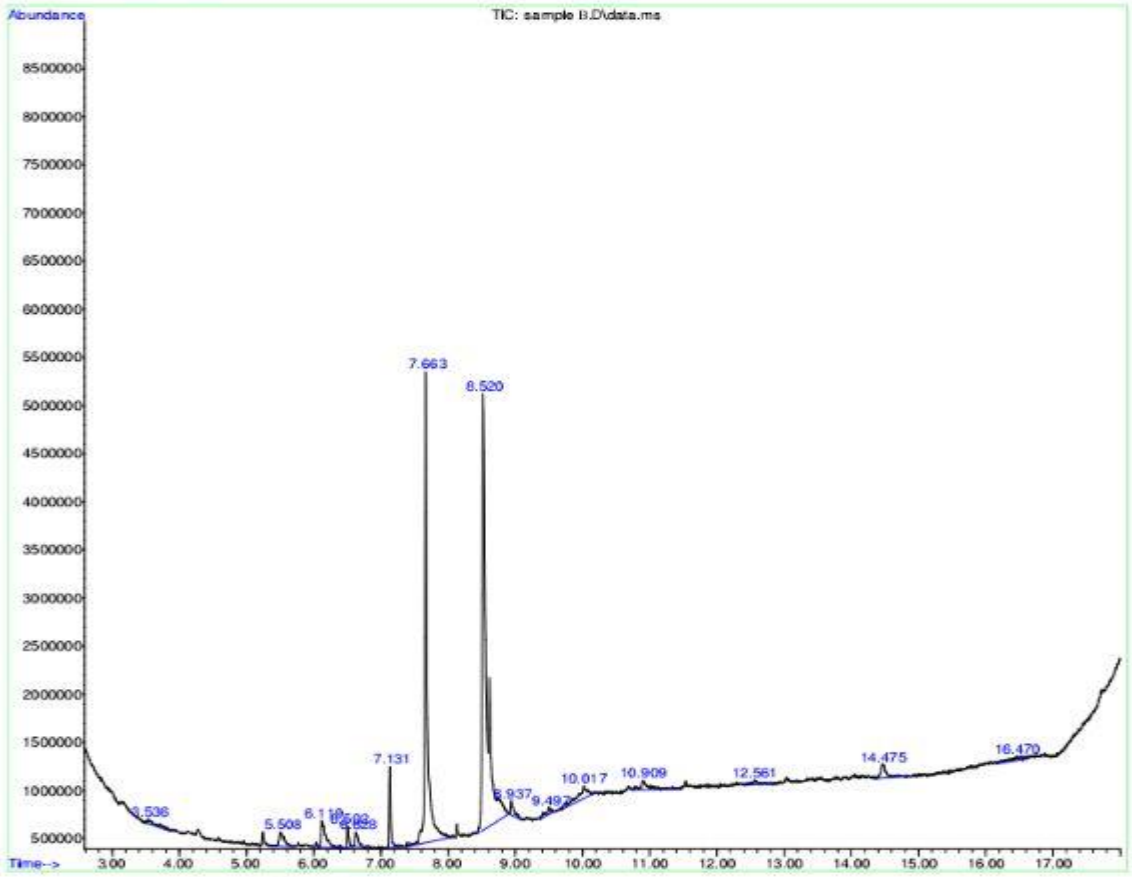
Appendix 4

Area Percent Report

Data Path : C:\msdchem\1\data\TEST 2\
Data File : sample B.D
Acq On : 3 Aug 2023 8:04
Operator : ADMIN 1
Sample : sample B
Misc :
ALS Vial : 2 Sample Multiplier: 1
Integration Parameters: rteiht.p
Integrator: RTE
Smoothing : ON Filtering: 5
Sampling : 5 Min Area: 1 Area counts
Start Thrs: 0.001 Max Peaks: 15
Stop Thrs : 0.01 Peak Location: TOP

If leading or trailing edge < 100 prefer < Tangent else baseline drop >
Peak separation: 1

Method : C:\msdchem\1\methods\Scan_.M
Title : PAH



Scan_.M Fri Aug 04 12:40:00 2023 Page: 2

Figure 4.1.2: SAMPLE B (Nano extract of *Hibiscus sabdariffa)* CHROMATOGRAPH

Appendix 5

Sample identification : S
Data measured at : 11-Dec-2023 7:22
Diffractometer type : PW 1800
Model : Rigaku D/Max-IIIC
Tube anode : Cu
Generator tension [kV] : 40
Generator current [mA] : 20
Wavelength Cuka 1[A^o] : 1.662
Wavelength Cuka 2[A^o] : 1.666
Intensity ratio (alpha2/alpha1): 0.500
Divergence slit : Fine
Irradiated length [mm] : 2
Receiving slit : Fine
Spinner : ON
Monochromator used : YES
Start angle [2^o] : 24.00
End angle [2^o] : 56.00
Step size [2^o] : 0.020
Maximum intensity : 2300cps
Time per step [s] : 0.250
Type of scan : CONTINUOUS
Intensities converted to : FIXED
Diffraction smoothed by : Savitsky-Golay
Heigth (Hc) at : 17^o
B type crystallinity peak approximate : $2q = 22^o$
Peak base width : 2.00
Minimum significance : 0.75
Number of peaks : 4

Peak no.	2theta	FWHM	d-value	Counts	I/Io
1	24.5	1.024	1.351	2300	12
2	30.4	0.031	2.250	10	14
3	32.6	1.121	2.023	800	10
4	45.4	1.131	2.213	10	9
5	57.1	1.164	3.487	800	6
6	60.5	0.173	3.586	10	26
7	65.6	0.128	2.339	10	10
8	65.6	0.148	2.719	10	10
9	69.6	0.121	4.023	10	12

XRD RESULT TABLE

Appendix 6: Statistical analysis

In-Vitro Free Radical Scavenging Assay Table

2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

		Sample A				*Sample B*			
	DPPH								
S/NO	Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)	OD1	OD2	Mean OD	Scavenging (%)
1	0	0.062	0.064	0.063	0.00	0.066	0.066	0.066	0.00
2	50	0.057	0.058	0.0575	8.73	0.059	0.058	0.0585	11.36
3	100	0.053	0.052	0.0525	16.67	0.051	0.054	0.0525	20.45
4	200	0.05	0.053	0.0515	18.25	0.045	0.044	0.0445	32.58
5	400	0.047	0.045	0.046	26.98	0.032	0.034	0.033	50.00
6	500	0.044	0.042	0.043	31.75	0.027	0.025	0.026	60.61
7	800	0.042	0.04	0.041	34.92	0.016	0.019	0.0175	73.48
8	1000	0.037	0.037	0.037	41.27	0.012	0.01	0.011	83.33
9	1500	0.033	0.035	0.034	46.03	0	0	0	100.00
10	2000	0.029	0.028	0.0285	54.76	0	0	0	100.00

Gallic acid

Conc (µg/ml)	OD1	OD2	Mean	Scavenging (%)
0	0.333	0.354	0.3435	0.00
5	0.298	0.327	0.3125	9.02
10	0.274	0.248	0.261	24.02
20	0.229	0.234	0.2315	32.61
50	0.197	0.194	0.1955	43.09
80	0.163	0.171	0.167	51.38
100	0.145	0.138	0.1415	58.81
150	0.102	0.105	0.1035	69.87
250	0.062	0.052	0.057	83.41

500	0.013	0.015	0.014	95.92

Nitric oxide radical scavenging

	Sample A				*Sample B*			
Conc (µg/ml)	OD 1	OD2	Mean	Inhibition (%)	OD 1	OD2	Mean	Inhibition (%)
0	0.158	0.16	0.159	0.00	0.174	0.169	0.1715	0.00
50	0.144	0.16	0.152	4.40	0.168	0.167	0.1675	2.33
100	0.13	0.142	0.136	14.47	0.155	0.149	0.152	11.37
200	0.127	0.12	0.1235	22.33	0.127	0.13	0.1285	25.07
400	0.109	0.11	0.1095	31.13	0.115	0.113	0.114	33.53
800	0.097	0.095	0.096	39.62	0.102	0.098	0.1	41.69
1000	0.089	0.092	0.0905	43.08	0.093	0.096	0.0945	44.90
2000	0.085	0.087	0.086	45.91	0.076	0.08	0.078	54.52
2500	0.079	0.08	0.0795	50.00	0.066	0.072	0.069	59.77
4000	0.073	0.064	0.0685	56.92	0.052	0.055	0.0535	68.80

	Quercetin			
Conc (µg/ml)	OD 1	OD 2	Mean OD	% Inhibition
0	0.212	0.203	0.2075	0.000
10	0.185	0.164	0.1745	15.904
20	0.131	0.126	0.1285	38.072
50	0.109	0.114	0.1115	46.265
100	0.097	0.092	0.0945	54.458
150	0.088	0.086	0.087	58.072
250	0.069	0.062	0.0655	68.434
500	0.051	0.042	0.0465	77.590
800	0.018	0.018	0.018	91.325
1000	0.007	0.007	0.007	96.627

Hydroxyl radical scavenging

	SAMPLE A				*SAMPLE B*			
Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)	OD1	OD2	Mean OD	Scavenging (%)
0	0.225	0.223	0.224	0.00	0.214	0.234	0.224	0.00
50	0.218	0.214	0.216	3.57	0.195	0.21	0.2025	9.60
100	0.193	0.19	0.1915	14.51	0.176	0.174	0.175	21.88
200	0.165	0.172	0.1685	24.78	0.15	0.155	0.1525	31.92
400	0.144	0.153	0.1485	33.71	0.122	0.124	0.123	45.09
800	0.132	0.13	0.131	41.52	0.116	0.102	0.109	51.34
1000	0.12	0.122	0.121	45.98	0.106	0.102	0.104	53.57
2000	0.116	0.117	0.1165	47.99	0.093	0.094	0.0935	58.26
2500	0.1	0.098	0.099	55.80	0.084	0.079	0.0815	63.62
4000	0.098	0.092	0.095	57.59	0.064	0.068	0.066	70.54

	Quercetin			
Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)
0	0.285	0.271	0.278	0.00
10	0.241	0.248	0.2445	12.05
20	0.172	0.18	0.176	36.69
50	0.117	0.113	0.115	58.63
100	0.088	0.09	0.089	67.99
150	0.052	0.049	0.0505	81.83
250	0.03	0.031	0.0305	89.03
500	0.017	0.018	0.0175	93.71
800	0.005	0.008	0.0065	97.66
1000	0.005	0.001	0.003	98.92

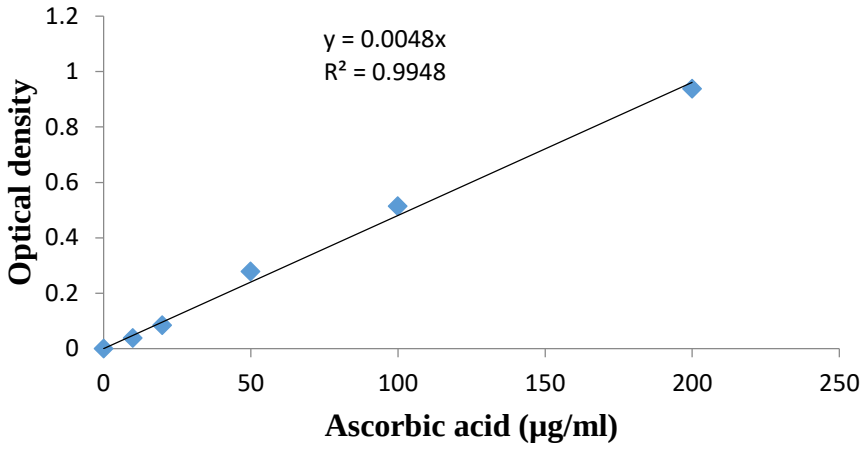
Super oxide radical scavenging

	SAMPLE A					*SAMPLE B*			
Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)	Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)
0	0.34	0.377	0.3585	0.00	0	0.346	0.354	0.35	0.00
50	0.318	0.317	0.3175	11.44	50	0.317	0.321	0.319	8.86
100	0.293	0.29	0.2915	18.69	100	0.295	0.294	0.2945	15.86
200	0.25	0.242	0.246	31.38	200	0.25	0.267	0.2585	26.14
400	0.216	0.215	0.2155	39.89	400	0.22	0.234	0.227	35.14
800	0.183	0.183	0.183	48.95	800	0.176	0.182	0.179	48.86
1000	0.152	0.147	0.1495	58.30	1000	0.156	0.152	0.154	56.00
2000	0.128	0.125	0.1265	64.71	2000	0.132	0.134	0.133	62.00
2500	0.109	0.099	0.104	70.99	2500	0.094	0.12	0.107	69.43
4000	0.088	0.075	0.0815	77.27	4000	0.084	0.088	0.086	75.43

	Quercetin			
Conc (µg/ml)	OD1	OD2	Mean OD	Scavenging (%)
0	0.325	0.341	0.333	0.00
10	0.322	0.324	0.323	3.00
20	0.298	0.318	0.308	7.51
50	0.247	0.213	0.23	30.93
100	0.188	0.19	0.189	43.24
150	0.172	0.169	0.1705	48.80
250	0.145	0.131	0.138	58.56
500	0.117	0.118	0.1175	64.71
800	0.1	0.098	0.099	70.27
1000	0.075	0.071	0.073	78.08

Total antioxidant capacity

	Ascorbic acid		
Conc (ug/ml)	OD1	OD2	Mean OD
0	0	0	0
10	0.037	0.039	0.038
20	0.073	0.095	0.084
50	0.297	0.26	0.2785
100	0.512	0.516	0.514
200	0.928	0.945	0.9365
400	1.201	1.188	1.1945
500	1.236	1.225	1.2305



Total antioxidant capacity calibration curve

	OD	*TAC (AAE ug/ml)*	*Conc (g/ml)*	*TAC (mg AAE /g extract)*
Sampe A	0.250	0.245	0.004	61.30
	0.256	0.251	0.004	62.80
	0.264	0.259	0.004	64.80
	0.248	0.243	0.004	60.80
Sample B	0.390	0.385	0.004	96.30
	0.330	0.325	0.004	81.30
	0.336	0.331	0.004	82.80
	0.364	0.359	0.004	89.80

ONEWAY DPPH BY FreeRadicalScavengingEffectOfNanoParticleAndExtract
/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS
/POSTHOC=TUKEY ALPHA(0.05).

Oneway

Notes

Output Created

Comments

Input Active Dataset

Filter

Weight

Split File

N of Rows in Working Data File

Missing Value Handling Definition of Missing

Cases Used

Syntax

Resources Processor Time

Elapsed Time

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User-defined missing values are treated as missing.

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ONEWAY DPPH BY FreeRadicalScavengingEff ectOfNanoParticleAndExtr act /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=TUKEY ALPHA(0.05).

00:00:00.05

00:00:00.17

Page 1

Descriptives

DPPH DPPH DPPH

N Mean Std. Deviation Std. Error

95% Confidence ...

Lower Bound

Sample A

Sample B

GALLIC ACID

Total

3 421.6800 13.92000 8.03672 387.1008 456.2592

3 6160.0000 49.28000 28.45182 6037.5817 6282.4183

3 213.8100 1.71000 .98727 209.5621 218.0579

9 2265.1633 2922.62622 974.20874 18.6339 4511.6927

DPPH DPPH

Descriptives

DPPH DPPH DPPH

95% Confidence Interval for Mean

Minimum MaximumUpper Bound

Sample A

Sample B

GALLIC ACID

Total

456.2592 407.76 435.60

6282.4183 6110.72 6209.28

218.0579 212.10 215.52

4511.6927 212.10 6209.28

DPPH DPPH

Test of Homogeneity of Variances

Levene Statistic df1 df2 Sig.

DPPH Based on Mean

Based on Median

Based on Median and with adjusted df

Based on trimmed mean

2.790 2 6 .139

2.790 2 6 .139

2.790 2 2.322 .241

2.790 2 6 .139

ANOVA

DPPH DPPH DPPH

Sum of Squares df Mean Square F Sig.

Between Groups

Within Groups

Total

68328701.88 2 34164350.94 39041.866 .000

5250.418 6 875.070

68333952.29 8

DPPH DPPH

Post Hoc Tests

Page 2

Multiple Comparisons

Dependent Variable: DPPH Dependent Variable: DPPH

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

Mean Difference (I-J) Std. Error Sig.

Sample A Sample B

GALLIC ACID

Sample B Sample A

GALLIC ACID

GALLIC ACID Sample A

Sample B

-5738.32000* 24.15326 .000 -5812.4288 207.87000* 24.15326 .000 133.7612 5738.32000* 24.15326 .000 5664.2112 5946.19000* 24.15326 .000 5872.0812 -207.87000* 24.15326 .000 -281.9788 -5946.19000* 24.15326 .000 -6020.2988

Dependent Variable: DPPH

Tukey HSD

Multiple Comparisons

Dependent Variable: DPPH Dependent Variable: DPPH

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

95% Confidence Interval

Lower Bound Upper Bound

Sample A Sample B

GALLIC ACID

Sample B Sample A

GALLIC ACID

GALLIC ACID Sample A

Sample B

-5812.4288 -5664.2112

133.7612 281.9788

5664.2112 5812.4288

5872.0812 6020.2988

-281.9788 -133.7612

-6020.2988 -5872.0812

Dependent Variable: DPPH

Tukey HSD

The mean difference is significant at the 0.05 level.*.

Homogeneous Subsets

DPPH

Tukey HSDa Tukey HSDa Tukey HSDa

FreeRadicalScavengingEffe ctOfNanoParticleAndExtract N

Subset for alpha = 0.05

1 2 3

GALLIC ACID

Sample A

Sample B

Sig.

3 213.8100

3 421.6800

3 6160.0000

1.000 1.000 1.000

Tukey HSDa Tukey HSDa

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.a.

Page 3

```
ONEWAY NITRICOXIDE BY FreeRadicalScavengingEffectOfNanoParticleAndExtract
/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS
/POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

Notes

Output Created

Comments

Input Active Dataset

Filter

Weight

Split File

N of Rows in Working Data File

Missing Value Handling Definition of Missing

Cases Used

Syntax

Resources Processor Time

Elapsed Time

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ONEWAY NITRICOXIDE BY FreeRadicalScavengingEff ectOfNanoParticleAndExtr act /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=TUKEY ALPHA(0.05).

00:00:00.06

00:00:00.08

Page 4

Descriptives

NITRICOXIDE NITRICOXIDE NITRICOXIDE NITRICOXIDE

N Mean Std. Deviation Std. Error

95% Confidence Interval for Mean

Lower Bound Upper Bound

Sample A

Sample B

QUERCETIN

Total

3 381.9600 4.20000 2.42487 371.5266 392.3934 377.76

3 720.8200 10.81000 6.24116 693.9665 747.6735 710.01

3 609.1600 10.98000 6.33931 581.8842 636.4358 598.18

9 570.6467 149.75981 49.91994 455.5311 685.7622 377.76

NITRICOXIDE NITRICOXIDE

Descriptives

NITRICOXIDE NITRICOXIDE NITRICOXIDE

Minimum Maximum

Sample A

Sample B

QUERCETIN

Total

377.76 386.16

710.01 731.63

598.18 620.14

377.76 731.63

NITRICOXIDE NITRICOXIDE

Test of Homogeneity of Variances

Levene Statistic df1 df2 Sig.

NITRICOXIDE Based on Mean

Based on Median

Based on Median and with adjusted df

Based on trimmed mean

.703 2 6 .532

.703 2 6 .532

.703 2 4.565 .542

.703 2 6 .532

ANOVA

NITRICOXIDE NITRICOXIDE NITRICOXIDE

Sum of Squares df Mean Square F Sig.

Between Groups

Within Groups

Total

178913.895 2 89456.948 1052.202 .000

510.113 6 85.019

179424.008 8

NITRICOXIDE NITRICOXIDE

Post Hoc Tests

Page 5

Multiple Comparisons

Dependent Variable: NITRICOXIDE Dependent Variable: NITRICOXIDE

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

Mean Difference (I-J) Std. Error Sig.

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

-338.86000* 7.52856 .000 -361.9597 -227.20000* 7.52856 .000 -250.2997 338.86000* 7.52856 .000 315.7603 111.66000* 7.52856 .000 88.5603 227.20000* 7.52856 .000 204.1003 -111.66000* 7.52856 .000 -134.7597

Dependent Variable: NITRICOXIDE

Tukey HSD

Multiple Comparisons

Dependent Variable: NITRICOXIDE Dependent Variable: NITRICOXIDE

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

95% Confidence Interval

Lower Bound Upper Bound

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

-361.9597 -315.7603

-250.2997 -204.1003

315.7603 361.9597

88.5603 134.7597

204.1003 250.2997

-134.7597 -88.5603

Dependent Variable: NITRICOXIDE

Tukey HSD

The mean difference is significant at the 0.05 level.*.

Homogeneous Subsets

NITRICOXIDE

Tukey HSDa Tukey HSDa Tukey HSDa

FreeRadicalScavengingEffe ctOfNanoParticleAndExtract N

Subset for alpha = 0.05

1 2 3

Sample A

QUERCETIN

Sample B

Sig.

3 381.9600

3 609.1600

3 720.8200

1.000 1.000 1.000

Tukey HSDa Tukey HSDa

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.a.

Page 6

ONEWAYHYDROXYLRADICAL BY FreeRadicalScavengingEffectOfNanoParticleAndExtra
ct

/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

Oneway

Notes

Output Created

Comments

Input Active Dataset

Filter

Weight

Split File

N of Rows in Working Data File

Missing Value Handling Definition of Missing

Cases Used

Syntax

Resources Processor Time

Elapsed Time

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User-defined missing values are treated as missing.

Statistics for each analysis are based on cases with no missing data for any variable in the analysis.

ONEWAY HYDROXYLRADICAL BY FreeRadicalScavengingEff ectOfNanoParticleAndExtr act /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=TUKEY ALPHA(0.05).

00:00:00.05

00:00:00.13

Page 7

Descriptives

HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL

N Mean Std. Deviation Std. Error

95% Confidence Interval for Mean

Lower Bound Upper Bound

Sample A

Sample B

QUERCETIN

Total

3 326.8900 3.92000 2.26321 317.1522 336.6278 322.97

3 282.1600 3.10000 1.78979 274.4592 289.8608 279.06

3 41.0200 .78000 .45033 39.0824 42.9576 40.24

9 216.6900 133.19258 44.39753 114.3091 319.0709 40.24

HYDROXYLRADICAL HYDROXYLRADICAL

Descriptives

HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL

Minimum Maximum

Sample A

Sample B

QUERCETIN

Total

322.97 330.81

279.06 285.26

40.24 41.80

40.24 330.81

HYDROXYLRADICAL HYDROXYLRADICAL

Test of Homogeneity of Variances

Levene Statistic df1 df2 Sig.

HYDROXYLRADICAL Based on Mean

Based on Median

Based on Median and with adjusted df

Based on trimmed mean

1.244 2 6 .353

1.244 2 6 .353

1.244 2 3.981 .380

1.244 2 6 .353

ANOVA

HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL HYDROXYLRADICAL

Sum of Squares df Mean Square F Sig.

Between Groups

Within Groups

Total

141870.929 2 70935.465 8317.688 .000

51.170 6 8.528

141922.099 8

HYDROXYLRADICAL HYDROXYLRADICAL

Post Hoc Tests

Page 8

Multiple Comparisons

Dependent Variable: HYDROXYLRADICAL Dependent Variable: HYDROXYLRADICAL

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

Mean Difference (I-J) Std. Error Sig.

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

44.73000* 2.38443 .000 37.4139 285.87000* 2.38443 .000 278.5539 -44.73000* 2.38443 .000 -52.0461 241.14000* 2.38443 .000 233.8239 -285.87000* 2.38443 .000 -293.1861 -241.14000* 2.38443 .000 -248.4561

Dependent Variable: HYDROXYLRADICAL

Tukey HSD

Multiple Comparisons

Dependent Variable: HYDROXYLRADICAL Dependent Variable: HYDROXYLRADICAL

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

95% Confidence Interval

Lower Bound Upper Bound

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

37.4139 52.0461

278.5539 293.1861

-52.0461 -37.4139

233.8239 248.4561

-293.1861 -278.5539

-248.4561 -233.8239

Dependent Variable: HYDROXYLRADICAL

Tukey HSD

The mean difference is significant at the 0.05 level.*.

Homogeneous Subsets

HYDROXYLRADICAL

Tukey HSDa Tukey HSDa Tukey HSDa

FreeRadicalScavengingEffe ctOfNanoParticleAndExtract N

Subset for alpha = 0.05

1 2 3

QUERCETIN

Sample B

Sample A

Sig.

3 41.0200

3 282.1600

3 326.8900

1.000 1.000 1.000

Tukey HSDa Tukey HSDa

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.a.

Page 9

ONEWAY SUPEROXIDERADICAL BY FreeRadicalScavengingEffectOfNanoParticleAndExt
ract
/STATISTICS DESCRIPTIVES HOMOGENEITY

/MISSING ANALYSIS
/POSTHOC=TUKEY ALPHA(0.05).

Oneway

Notes

Output Created

Comments

Input Active Dataset

Filter

Weight

Split File

N of Rows in Working Data File

Missing Value Handling Definition of Missing

Cases Used

Syntax

Resources Processor Time

Elapsed Time

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9

User-defined missing values are treated as missing.

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ONEWAY SUPEROXIDERADICAL BY FreeRadicalScavengingEff ectOfNanoParticleAndExtr act /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=TUKEY ALPHA(0.05).

00:00:00.03

00:00:00.07

Page 10

Descriptives

SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL

N Mean Std. Deviation Std. Error

95% Confidence Interval for Mean

Lower Bound Upper Bound

Sample A

Sample B

QUERCETIN

Total

3 668.8200 3.34000 1.92835 660.5230 677.1170 665.48

3 664.0700 2.65000 1.52998 657.4870 670.6530 661.42

3 87.5200 1.14000 .65818 84.6881 90.3519 86.38

9 473.4700 289.47822 96.49274 250.9573 695.9827 86.38

SUPEROXIDERADICAL SUPEROXIDERADICAL

Descriptives

SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL

Minimum Maximum

Sample A

Sample B

QUERCETIN

Total

665.48 672.16

661.42 666.72

86.38 88.66

86.38 672.16

SUPEROXIDERADICAL SUPEROXIDERADICAL

Test of Homogeneity of Variances

Levene Statistic df1 df2

SUPEROXIDERADICAL Based on Mean

Based on Median

Based on Median and with adjusted df

Based on trimmed mean

.780 2 6 .500

.780 2 6 .500

.780 2 4.325 .514

.780 2 6 .500

Test of Homogeneity of Variances

Sig.

SUPEROXIDERADICAL Based on Mean

Based on Median

Based on Median and with adjusted df

Based on trimmed mean

.500

.500

.514

.500

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ANOVA

SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL SUPEROXIDERADICAL

Sum of Squares df Mean Square F Sig.

Between Groups

Within Groups

Total

670342.155 2 335171.078 51623.818 .000

38.955 6 6.493

670381.110 8

SUPEROXIDERADICAL SUPEROXIDERADICAL

Post Hoc Tests

Multiple Comparisons

Dependent Variable: SUPEROXIDERADICAL Dependent Variable: SUPEROXIDERADICAL

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

Mean Difference (I-J) Std. Error Sig.

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

4.75000 2.08048 .135 -1.6335 581.30000* 2.08048 .000 574.9165

-4.75000 2.08048 .135 -11.1335 576.55000* 2.08048 .000 570.1665 -581.30000* 2.08048 .000 -587.6835 -576.55000* 2.08048 .000 -582.9335

Dependent Variable: SUPEROXIDERADICAL

Tukey HSD

Multiple Comparisons

Dependent Variable: SUPEROXIDERADICAL Dependent Variable: SUPEROXIDERADICAL

Tukey HSD Tukey HSD

(I) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

(J) FreeRadicalScavengingEffe ctOfNanoParticleAndExtract

95% Confidence Interval

Lower Bound Upper Bound

Sample A Sample B

QUERCETIN

Sample B Sample A

QUERCETIN

QUERCETIN Sample A

Sample B

-1.6335 11.1335

574.9165 587.6835

-11.1335 1.6335

570.1665 582.9335

-587.6835 -574.9165

-582.9335 -570.1665

Dependent Variable: SUPEROXIDERADICAL

Tukey HSD

The mean difference is significant at the 0.05 level.*.

Homogeneous Subsets

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SUPEROXIDERADICAL

Tukey HSDa Tukey HSDa Tukey HSDa

FreeRadicalScavengingEffe ctOfNanoParticleAndExtract N

Subset for alpha = 0.05

1 2

QUERCETIN

Sample B

Sample A

Sig.

3 87.5200

3 664.0700

3 668.8200

1.000 .135

Tukey HSDa Tukey HSDa

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000

```
T-TEST GROUPS=TotalAntioxidantCapacityofNanoAndExtract (1 2)
 /MISSING=ANALYSIS
 /VARIABLES=TAC
 /CRITERIA=CI(.95).
```

T-Test

Notes

Output Created

Comments

Input Active Dataset

Filter

Weight

Split File

N of Rows in Working Data File

Missing Value Handling Definition of Missing

Cases Used

Syntax

Resources Processor Time

Elapsed Time

07-NOV-2024 12:12:56

DataSet1

<none>

<none>

<none>

8

User defined missing values are treated as missing.

Statistics for each analysis are based on the cases with no missing or out-ofrange data for any variable in the analysis.

T-TEST GROUPS=TotalAntioxidantCapacityofNanoAndExtra ct(1 2) /MISSING=ANALYSIS /VARIABLES=TAC /CRITERIA=CI(.95).

00:00:00.03

00:00:00.05

[DataSet1]

Group Statistics

TotalAntioxidantCapacityof NanoAndExtract N Mean Std. Deviation Std. Error Mean

TAC Sample A

Sample B

4 62.4250 1.79699 .89849

4 87.5500 6.91014 3.45507

Page 1Independent Samples Test

Levene's Test for Equality of Variances

t-test for Equality of Means

F Sig. t df

TAC Equal variances assumed

Equal variances not assumed

8.377 .028 -7.038 6 .000

-7.038 3.404 .004

Independent Samples Test

t-test for Equality of Means

Sig. (2-tailed)

Mean Difference

Std. Error Difference

95% Confidence ...

Lower

TAC Equal variances assumed

Equal variances not assumed

.000 -25.12500 3.56998 -33.86044 -16.38956

.004 -25.12500 3.56998 -35.76041 -14.48959

Independent Samples Test

t-test for Equality of Means

95% Confidence Interval of the ...

Upper

TAC Equal variances assumed

Equal variances not assumed

-16.38956

-14.48959

Appendix 7

Results of the antimicrobial analysis of the crude and nano extract of *Hibiscus Sabdariffa* using spectrophotometric assay:

Table 1

Colonial and Microscopic Characteristics of Test Cultures

Colonial Characteristics	Spore Formation	Motility	Gram Reaction	Identity of Isolates
Small smooth moist and shiny golden yellow colonies on Nutrient agar and yellow colonies on MSA	-	-	+S	*Staphylococcus aureus*
Small circular moist and shiny pink colonies on MacConkey agar and greenish metallic sheen on EMBA	-	+	-R	*Escherichia coli*
Smooth and shiny fish eye colonies on Salmonella Shigella agar	-	+	-R	*Salmonella* sp

R, rod shaped; S, spherical or cocci/round shaped

Table 2

Microscopic and Biochemical Characteristics of Test Cultures

NO_3 reduction	H_2S production	Ure	Oxi	Cat	Coag	In	MR	VP	S	L	G	M	Identity of isolates
+	-	-	-	+	+	-	-	+	+	+	+	+	*Staphylococcus aureus*
+	-	-	-	+	-	+	+	-	+	+	+	+	*Escherichia coli*
+	+	+	-	+	-	-	-	+	+	-	+	-	*Salmonella* sp

NO_3, nitrate reduction test; H_2S, hydrogen sulphide production test; Ure, Urease; Oxi, oxidase test; Cat, Catalase test; Coag, coagulase test; In, indole test; MR, Methyl Red test; VP, VogesProskaeur test; S, sucrose; L, lactose; G, glucose; M, maltose; nd, not done

Sensitivity of *Hibiscus Sabdariffa* Plant Extract on Test Organisms in Diameter (mm)

Test isolates	Sample code	500 mg/ml	250 mg/l	125 mg/l	62.5 mg/l	CIP
Staphylococcus aureus	A1	15	0	0	0	45
	A2	20	0	0	0	45
		()	()	()	()	()
Escherichia coli	A 1	20	0	0	0	40
	A2	18	0	0	0	42
		()	()	()	()	()
Salmonella sp	A1	25	20	15	0	45
	A2	20	18	20	0	45
		()	()	()	()	()

CIP, Ciprofloxacin

Calculate mean in bracket and determine standard deviation and standard error

Sensitivity of Silver nitrate Nano synthesized Plant Extracts on Test Organisms in Diameter (mm)

Test isolates	Sample code	500 mg/ml	250 mg/l	125 mg/l	62.5 mg/l	CIP
Staphylococcus aureus	B1	25	35	25	20	45
	B2	30	30	20	30	40
		()	()	()	()	()
Escherichia coli	B 1	22	20	15	20	40
	B2	30	25	20	15	35
		()	()	()	()	()
Salmonella sp	B1	30	30	25	20	45
	B2	35	25	20	20	45

		()	()	()	()	()

CIP, Ciprofloxacin

Table 3

Minimum Inhibitory Concentration (MIC) of Plant Extracts on Test isolates (λ=430 nm)

BEFORE INCUBATION

Hibiscus Sabdariffa Plant Extract	OD of Medium for Standardization	Test Organisms	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml
A	0.012	*Escherichia coli*	0.173 0.178 ()	0.128 0.126 ()	0.066 0.072 ()	0.043 0.049 ()
	0.012	*Staphylococcus aureus*	0.121 0.120 ()	0.072 0.081 ()	0.032 0.030 (0)	0.022 0.031 (0)
	0.012	*Salmonella* sp	0.093 0.094 ()	0.067 0.077 ()	0.057 0.055 ()	0.041 0.048 ()

Calculate mean in bracket and determine standard deviation and standard error

Table 4

Minimum Inhibitory Concentration (MIC) of Plant Extracts on Test isolates (λ=430 nm)

AFTER INCUBATION

Hibiscus Sabdariffa Plant Extract	OD of Medium for Standardization	Test Organisms	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml
A	0.012	*Escherichia coli*	0.056 0.051 ()	0.132 0.130 ()	0.077 0.079 ()	0.047 0.050 ()
	0.012	*Staphylococcus aureus*	0.044 0.052 ()	0.070 0.071 ()	0.081 0.087 (0)	0.190 0.100 (0)

	0.012	*Salmonella* sp	0.031 0.044 ()	0.059 0.051 ()	0.077 0.085 ()	0.064 0.060 ()

Calculate mean in bracket and determine standard deviation and standard error

Table 5

Minimum Bactericidal Concentration (MBC) of Plants Extract (A) on Test Organisms

Plant Extract	Bacterial Isolates	MBC of Extract (mg/ml)
A	*Escherichia coli*	500
	Staphylococcus aureus	250
	Salmonella sp	500

Table 6

Minimum Inhibitory Concentration (MIC) of the Nano Extract on Test isolates (ƛ=430 nm)

BEFORE INCUBATION

Silver nitrate Nano-synthesized Plant Extract	OD of Medium for Standardization	Test Organisms	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml
B	0.012	*Escherichia coli*	0.110 0.111 ()	0.099 0.095 ()	0.077 0.083 ()	0.055 0.051 ()
	0.012	*Staphylococcus aureus*	0.098 0.091 ()	0.061 0.066 ()	0.040 0.044 (0)	0.033 0.030 (0)
	0.012	*Salmonella* sp	0.084 0.080 ()	0.050 0.055 ()	0.034 0.037 ()	0.022 0.024 ()

Calculate mean in bracket and determine standard deviation and standard error

Table 7

Minimum Inhibitory Concentration (MIC) of Nano Extract on Test isolates (ƛ=430 nm)

AFTER INCUBATION

Silver nitrate Nano-synthesized Plant Extract	OD of Medium for Standardization	Test Organisms	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml
B	0.012	*Escherichia coli*	0.021 0.023 ()	0.166 0.166 ()	0.051 0.055 ()	0.037 0.041 ()
	0.012	*Staphylococcus aureus*	0.029 0.031 ()	0.042 0.047 ()	0.050 0.057 (0)	0.055 0.056 (0)
	0.012	*Salmonella* sp	0.021 0.020 ()	0.038 0.033 ()	0.054 0.050 ()	0.049 0.053 ()

Calculate mean in bracket and determine standard deviation and standard error

Table 9

Minimum Bactericidal Concentration (MBC) of Plant Extract on Test Organisms

Plant Extract	Bacterial Isolates	MBC of Extract (mg/ml)
B	*Escherichia coli*	500
	Staphylococcus aureus	250
	Salmonella sp	500