

Synthesis and Characterization of ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite Nanocomposites for Antibacterial and Antifungal Applications



Sisay Geda Bekele

A Thesis Submitted to

The Department of Applied Chemistry

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master of Science in
Applied Chemistry (In Inorganic Chemistry)

Office of Graduate Studies

Adama Science and Technology University

June, 2023

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master thesis entitled “Synthesis and Characterization of ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite Nanocomposites for Antibacterial and Antifungal Applications” is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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We, the advisor(s) of this thesis, hereby certify that we have read the revised version of the thesis entitled “Synthesis and Characterization of ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite Nanocomposites for Antibacterial and Antifungal Applications” prepared under our guidance by Sisay Geda Bekele submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry (In Inorganic Chemistry). Therefore, we recommend the submission of a revised version of the thesis to the department following the applicable procedures.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BT	Bentonite
CEC	Cation Exchange Capacity
DMSO	Dimethyl Sulfoxide
FTIR	Fourier Transforms Infrared Spectroscopy
FDA	Food And Drug Administration
IONPS	Iron Oxide Nanoparticles
NCs	Nanocomposites
NPs	Nanoparticles
MBC	Minimum Bacterial Concentration
MFC	Minimum Fungal Concentration
PL	Photoluminescence Spectroscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TGA	Thermo Gravimetric Analysis
UV-vis DRS	Ultraviolet-Visible Diffuse Reflectance Spectroscopy
XRD	X-Ray Diffraction Spectroscopy

ABSTRACT

Infectious diseases caused by bacteria and fungi pose significant global health challenges, and the emergence of drug-resistant bacterial strains has become a serious threat human health. To address this issue, the present study aimed to ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite nanocomposites using Hagenia abyssinica green method as antifungal and antibacterial agents. The study compared the antibacterial and antifungal efficacy of various ratios of synthesized Nanoparticles (NPs) and Nanocomposites (NCs) through green route. The synthesized materials were characterized using various techniques, including TGA, XRD, SEM, TEM, XPS, UV-DRS, FTIR, and CEC. XRD analysis confirmed the formation of α -Fe₂O₃ (spinel structure) and ZnO hexagonal phase (wurtzite structure) with different ratios. The XRD patterns of the primary, binary, and ternary nanocomposites confirmed the formation of (1:2), (1:1) α -Fe₂O₃ NPs and (1:2), (1:1), and (2:1) ZnO NPs, (1:1), (1:2) and (2:1) α -Fe₂O₃ /Bentonite, (1:1), (1:2) and (2:1) ZnO/Bentonite, and (1:1:1), (1:1:3) and (2:2:1) ZnO / α -Fe₂O₃/Bentonite NCs. The average crystalline sizes of these NPs and NCs were estimated to be about 10.36-30.1 nm, depending on the composition. UV-Vis DRS confirmed the band gap energy of synthesized NPs and NCs, ranging from 2.00-3.87 eV. The study found that the (1:1:1) ZnO / α -Fe₂O₃ /Bentonite NCs exhibited the best antibacterial activity towards E. coli and S. aureus, with maximum values of 13 ± 0.3 , 16 ± 0.3 at a dosage of 10 mg/mL, compared to the positive control. The (1:1:1) α -Fe₂O₃/ZnO/Bentonite ratio also displayed the best antifungal activity against Candida albicans, with a maximum zone of inhibition of 13 mm at a concentration of 10 mg/mL. The observed Minimum inhibitor concentration (MIC), minimum bacterial concentration (MBC), and minimum fungal concentration (MFC) values for α -Fe₂O₃/ZnO/Bentonite NCs were 156.25 μ g/mL, 78.125 μ g/mL, and 39.06 μ g/mL respectively, for E. coli, S. aureus, and Candida albicans. The MBC and MFC results above 0.078 mg/mL showed no bacterial or fungal colony growth, indicating the potential of α -Fe₂O₃/ZnO/Bentonite as an effective antibacterial and antifungal agent.

Keywords: Composite, Bentonite, Minimum inhibitor concentration, α -Fe₂O₃, antibacterial agent.

CHAPTER 1

INTRODUCTION

1.1. Background of the Study

Nanoscience and nanotechnology have been interesting fields of research and gained much attention in the last two decades (Franco et al., 2022). In layman's terms, “the study of properties of materials at the nanoscale is termed as nano-science, whereas the fabrication and application of the nanostructures are termed as nanotechnology”. NPs can be defined as a microscopic particle that has a size falling between 1 to 100 nm (Afolalu et al., 2019). They play an essential role as a building block of nanotechnology. Nowadays, nano-science as well as nanotechnology are widely applied in different fields mainly in sensors, electronics, water purification, cosmetics, biomedical devices, pharmaceuticals, environmental remediation, catalysis, and material applications (Kalkidan Mamo et al., 2021).

The size, crystallinity, and morphology of the nanomaterials can greatly influence their catalytic, magnetic, electronic, and optical properties (Yaqoob, et al., 2020). Because of their capacity to survive difficult processing conditions, inorganic materials like metal and metal oxides have attracted attention recently (Bhushan et al., 2018; Gordon et al., 2011). Several have been conducted on two of these materials, iron oxide (α -Fe₂O₃) (Fouad et al., 2019) and zinc oxide (ZnO) (Jayachandran et al., 2021). Because of their advantageous traits, which include stability, magnetic properties, strong temperature resistance, long-lastingness, low toxicity, selectivity, and biocompatibility.

Researchers have found that α -Fe₂O₃ and ZnO NPs have many potential uses, such as treating malaria, inflammation, and bacterial and fungal infections, as well as having antioxidant, antiviral, and anticancer properties (Abbas et al., 2020a). These NPs have shown promising results in preventing the growth of various types of bacteria and fungi including those that are resistant to antibiotics. Metal oxide nanoparticles (MO NPs) have a larger surface area, allowing them to easily interact with microorganisms. This process affects the permeability of the cell membrane, gene expression, protein activation, and enzyme activation which ultimately caused

the cell membrane to lose its integrity, stop cellular metabolism, and result in cell death (Gold et al., 2018).

However, using pure NPs alone for various applications can be challenging due to several issues such as limited biological studies, poor stability, agglomeration between NPs, and potential effects on humans and the environment associated with their size. Researchers have found a solution to these problems by combining NPs with other environmentally friendly and stable minerals through a process called hybrid/intercalation (Hakimi et al., 2018). By using this method, various applications of NPs can be improved. For example, mixing NPs with materials like bentonite (Rihayat et al., 2018), kaolinite (Siyao et al., 2022), and nano clay (Ngcobo et al., 2022) can reduce aggregation and improve the stability of the NPs. Also, these nanoparticles become hazardous to the environment when released in the form of large aggregates. Therefore, the choice of suitable capping moieties is key in stabilizing the colloidal solutions and their uptake into living cells and the environment. The surface chemistry and size distribution of nanoparticles get altered after capping with biocompatible (Marcos Luciano Bruschi & Lucas de Alcântara Sica de Toledo, 2019).

A useful substance known for reducing agglomeration in Metal oxide is bentonite (BT). It possesses a high cation exchange capacity, water dispersibility, swelling factor, and solid surface area (Khansili & Murali Krishna, 2021), all of which stabilize the NPs on its layered structure and stops ZnO and α -Fe₂O₃ nanomaterials from adhering together. This increases the number of active sites on their composite surfaces, which is more important than depending only on NPs. Due to large surface area and negative charge; bentonite has been studied for its potential antibacterial and antifungal properties. These unique properties allow to attract and bind with positively charged bacteria making it a promising material to combat microbial infections (Guo et al., 2018).

Metal oxide nanoparticles (MO NPs) can be synthesized through various methods, such as physical, biological and chemical. Green synthesis, in particular, is becoming popular due to its low cost, environmental compatibility, and lack of toxicity compared to other methods. Plant extracts are great sources of enzymes and phytochemical that react with metal salts to form MO NPs precursors quickly which make them to be excellent choices for green synthesis (Naaz et al., 2021). The MO NPs, synthesized using green synthesis, have been found to combat antibiotic-

resistant bacteria and fungi while reducing their environmental effects. The synthesizing NPs using plant extracts is also being explored for their photo-degradation, antibacterial, and antifungal properties (Bekele et al., 2021).

Many reports have been published in the literature on the green synthesis of α -Fe₂O₃ NPs and ZnO NPs using several plants extracts. The reducing property of different plant constituents plays a critical role in the reduction of Fe³⁺ and Zn²⁺ ions to α -Fe₂O₃ NPs and ZnO NPs and the stabilization of α -Fe₂O₃ NPs and ZnO NPs. To the best of my knowledge, there is no work reported on the synthesis, characterization, antibacterial and antifungal activities of α -Fe₂O₃ NPs, α -Fe₂O₃/Bent, and ZnO / α -Fe₂O₃/Bent using the extracts of *Hagenia abyssinica* medicinal plant.

Keeping these in view, the current study aims to develop a simple, non-toxic, cost-effective, and eco-friendly approach for the synthesis of ZnO NPs, α -Fe₂O₃NPs, ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/bentonite NCs using aqueous extract of *Hagenia abyssinica* leaf and characterization of synthesized NPs and NCs using UV-DRS, FT-IR, and XRD, SEM, TGA, and PL techniques. Further, the antibacterial activity of synthesized ZnO NPs, α -Fe₂O₃NPs, ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/bentonite NCs has been analyzed against human pathogens, Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria by using agar well diffusion assay method to reveal the functional utility of synthesized NCs in biomedical applications. Furthermore, antifungal activity of ZnO NPs, α -Fe₂O₃NPs, ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/bentonite NCs have also been evaluated against *candida albicans*.

1.2. Statement of the problem

Microbes such as bacteria and fungi cause infectious diseases that pose significant global health concerns, impacting not only human well-being but also leading to economic and social problems. These diseases are particularly troublesome in developing nations, where they can pose serious health risks, especially for vulnerable groups like children and the elderly. Meanwhile, culturally use medicines are becoming less effective as these microbes develop resistance to antimicrobial drugs. This growing resistance makes it increasingly difficult to control such diseases, and people with resistant infections may struggle to access or afford effective medications. This is especially problematic in developing nations, where there is a lack of suitable vaccines. To combat this critical issue, researchers around the world are turning to

metal and MO NPs as potential solutions. These tiny particles have shown impressive promise in fighting against the harmful effects of pathogenic microorganisms, providing a new avenue for developing treatments that can combat even the most difficult of infections. By applying the power of MO NPs; researchers hope to create effective treatments that can help to reduce the impact of infectious diseases, both in developing nations and across the globe. However, using pure NPs have several limitations that make them challenging to use for various applications, including limited biological studies, poor stability, agglomeration between NPs, and potential health and environmental risks associated with their size.

To overcome these challenges, natural clay, such as bentonite, can be used to adsorb ZnO and α -Fe₂O₃ MO NPs, enhancing their capabilities as antimicrobial agents. Bentonite has been found to prevent the aggregation, dissolution, and toxicity of these nanoparticles while also improving their stability, dispersibility, and interaction with fungal and bacterial cells. Furthermore, the combination of ZnO NPs with α -Fe₂O₃ and bentonite can increase the band gap of ZnO NPs. The increase in band gap leads to a higher production of reactive oxygen species, which can cause harm to the DNA and cell membranes of microorganisms by reducing the recombination of electron pairs. By combining these materials, researchers can look for more effective and stable antimicrobial agents with reduced environmental and health risks. However, the potential of ZnO/ α -Fe₂O₃/Bentonite nanocomposites in antibacterial and antifungal applications has not yet been explored. As a result, this study aimed to synthesize and characterize ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/bentonite for use in antibacterial and antifungal applications.

1.3. Objectives of the Study

1.3.1. General objective

The main objective of this study is to synthesize, characterize and evaluate the antibacterial and antifungal activities of ZnO/bentonite, α -Fe₂O₃/Bentonite, and ZnO / α -Fe₂O₃ /Bentonite NCs using aqueous extract of *H. abyssinica* leaf.

1.3.2. Specific objectives

The specific objectives of this work are:

- ✚ To synthesize α -Fe₂O₃/Bentonite, ZnO/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs via precipitation methods.
- ✚ To characterize synthesized NCs using UV-vis DRS, XRD, SEM, PL, FTIR, and TGA
- ✚ To evaluate antibacterial activities of ZnO, α -Fe₂O₃, α -Fe₂O₃/Bentonite, ZnO/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs against gram-positive bacteria (*S. aureus*) and gram-negative bacterial (*E. coli*) species
- ✚ To evaluate antifungal activities of the synthesized NPs and NCs against *candida albicans*.

1.4. Significance of the study

This study was designed to synthesize a metal oxide /bentonite clay binary and ternary NCs material and evaluate their antibacterial and antifungal activities. The significance of this study lay on the utilization of cost-effective, less toxic, and easily accessible material for the synthesis of metal oxide/bentonite clay NCs that ultimately ensure a healthy environment. Besides, this study was also expected to add knowledge about the synthesis of ZnO/bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs. The results of this study hold great potential for the fields of inorganic chemistry and material chemistry, and could contribute significantly to efforts aimed at addressing the challenge of drug resistance in microorganisms.

1.5. Scope of the study

The scope of this research was limited to the synthesis, and characterization of ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/bentonite NCs. Furthermore, a preliminary study was carried out on the composite suspension for its antibacterial and antifungal application against.

CHAPTER 2

LITERATURE REVIEW

2.1. Nanoparticles

Nanoparticles are particles within the nanoscale size between 1-100 nm with a surrounding interfacial layer. Nanotechnology is emerging as a promising technology, which has demonstrated remarkable feats in various fields including wastewater treatment and photocatalysis. Nanomaterials encompass a high surface-to-volume ratio, high sensitivity and reactivity, a high adsorption capacity, shape, charge, size, and ease of functionalization which makes them suitable for application in antimicrobial treatment (Faisal et al., 2022).

Investigations showed that the NPs size is the significant parameter that determined the antimicrobial effects of those materials. Bacterial cell walls attract NPs to their surface due to electrostatic interactions. These NPs establish a robust bond with membranes, leading to increased cell wall permeability. Metal NPs and metal ions, released from NPs, are capable of entering the cell and disrupting cell functions by inducing oxidative stress and suppressing the antioxidant defense reaction of bacteria against reactive oxygen species. Then, these ions are liberal to interact with cellular structures and form strong and non-specific coordination bonds with N, O, or S atoms of organic compounds which end up disrupting biological processes in a wide selection of microorganisms (Franco et al., 2022).

2.2. Metal oxide nanoparticles

Metallic oxide NPs (MO NPs) are the tiny powerhouses of materials science, measuring between 1-100 nm in width, thickness, and length. With their impressive physical, chemical, and biological properties, they have become the darling of various industries. MO NPs are versatile materials with a wide range of applications, including catalysis, sensors, electronic materials, and environmental remediation (Franco et al., 2022; Munonde & Nomngongo, 2021; Slavin & Bach, 2022). The unique properties of MONPs make them a promising material for a wide range of applications. For instance, in the field of electronics, they are used to fabricate electronic devices due to their electrical conductivity, high surface area, and size-dependent properties. In catalysis, MONPs are used for their high catalytic activity, selectivity, and stability. In energy, they are used in solar cells, fuel cells, and batteries due to their ability to absorb and transfer energy

efficiently. In the biomedical field, they are used for drug delivery, imaging, and therapy due to their small size, bio-compatibility, and ability to target specific cells or tissues (Zhu et al., 2013).

2.2.1. Iron oxide nanoparticles

Iron oxide NPs have gained attention in various applications due to their exceptional properties compared to their bulk counterparts (Fouad et al., 2019). Iron oxide-based nanoparticles have unique properties such as magnetic properties, catalytic activity, and high adsorption capacity for several contaminants in water and wastewater treatment. They are widely used in various biomedical applications including molecular imaging, hyperthermia treatment, protein separation, and drug delivery. Iron oxides are biocompatible MO NPs that have exceptional physical properties, including super-paramagnetic, firmness in liquid solutions, low susceptibility to oxidation, long blood half-lives, and flexible surface chemistry (Saied et al., 2022).

Magnetic NPs can damage bacteria by interfering with the thiol group at the respiratory base of these microorganisms (Caciandone et al., 2022). Various physical, chemical, and biological methods have been adopted to synthesize magnetic NPs with suitable surface chemistry. Recent research has focused on the synthesis, characterization, and applications of iron NPs, including their use in wastewater treatment and environmental and biomedical applications (Kulik et al., 2023). Among the various metal oxide (MO) nanomaterials, magnetic NPs have attracted a surge in interest due to their promising applications in drug delivery, hyperthermia treatment, bio-sensors, and bio electronics. Different forms of iron oxides (FeO , Fe_2O_3 , and Fe_3O_4) in different phases ($\alpha\text{-Fe}_2\text{O}_3$, $\beta\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$) have numerous fundamental, technological, and medical application(Rufus et al., 2016).

$\alpha\text{-Fe}_2\text{O}_3$ is known by a common name as hematite is recognized as the most thermodynamically stable form of iron oxide and has been extensively studied due to its low cost, less-toxicity and unique properties, which differ from its bulk form (Havenga et al., 2022). The $\alpha\text{-Fe}_2\text{O}_3$ is the most stable polymorph of iron oxide and is an ideal material for biomedical applications due to its adaptable magnetic and optical characteristics, great chemical stability, and biocompatibility. It is also the most common form of iron oxide found in nature as a mineral, occurring widely in rocks and soils (Vihodceva et al. 2021).

The α -Fe₂O₃ is an n-type semiconductor with a corundum crystal structure and is easier to synthesize than other forms of oxide, as it is the end product of other iron oxide transformations. Due to its α -Fe₂O₃ has small band gap (2.0-2.2 eV), it exhibits weak ferromagnetic or antiferromagnetic properties (Fouad et al., 2019). Various techniques are available to prepare α -Fe₂O₃ nanoparticles, including forced hydrolysis, hydrothermal technique, micro-emulsion technique, sol-gel process, template-assisted synthesis method, vapor-solid growth technique, high energy ball milling, and thermal decomposition technique (Pourmadadi et al., 2022). The crystal structure of hematite consists of iron atoms surrounded by six oxygen atoms in a hexagonal arrangement. Fe³⁺ cations fill two-thirds of the hexagonal interstices in the (001) basal planes, with the tetrahedral sites remaining vacant. Oxide ions (O²⁻) are found along the (001) plane in a hexagonal closed-packed lattice, resulting in the formation of FeO₆ octahedron pairs.

Hematite is paramagnetic and often found as a highly crystalline material in nature (Efficiencies et al., 2022). Recently, α -Fe₂O₃ is also used for drug delivery systems that can be directed by an external magnetic field for accurate treatment. However, the release of NPs into the environment can cause changes in their surface properties, resulting in particle aggregation or changes in particle charge. Surface modification is a method to improve the surface characteristics of α -Fe₂O₃NPs, prevent aggregation, enhance their compatibility with the biological environment, and improve stability in suspensions. Additionally, α -Fe₂O₃ is environmentally friendly and has diverse applications in water treatment, gas sensors, photocatalysis, and water splitting for H₂ generation (Kareem et al., 2018).

2.2.2. Zinc oxide Nanoparticles

Zinc oxide nanoparticles (ZnO NPs) are versatile materials with unique physical and chemical properties that make them attractive for a wide range of applications. ZnO belongs to the semiconductor group II-VI possesses a covalent character that falls between ionic and covalent semiconductors. It is a naturally n-type direct band gap semiconductor with a high electron-hole binding energy, and excellent photocatalytic and antibacterial activities. ZnO NPs can block a broad spectrum of wavelengths and provide anti-inflammatory activity due to their stable nature and selective targeting (Siddiqi et al., 2018). Various methods can be used to synthesize ZnO NPs, including physical, chemical, and biological methods. ZnO NPs can be applied in various fields, including antibacterial, pharmaceutical, and cosmetic applications. It is

considered the safest NPs recognized by the FDA-USA. ZnO NPs have the potential to be used as an antibacterial and antifungal agent to stop bacterial and fungi growth in pharmaceutical and food packaging. They are sensitive to both Gram-positive and Gram-negative bacteria and fungi with low toxicity toward human cells (Agarwal & Shanmugam, 2019).

The toxicity mechanisms of ZnO NPs towards bacteria or fungi mainly depend on factors such as particle size, concentration in suspension, the type of bacteria under investigation, the presence of surface modifiers, the availability of light, and the synthesis method employed. The antibacterial and antifungal mechanism of action of ZnO NPs is thought to involve physical and chemical interactions with the surface of bacteria and fungi, leading to the deposition of NPs, abrasion of the cell envelope and cytoplasm, or blocking of ion diffusion channels (Taufiq et al., 2018). ZnO NPs are a promising material with unique properties that make them attractive for various applications. However, their effectiveness depends on factors such as particle size, concentration, and synthesis method. Further research is needed to explore their potential applications and optimize their properties for specific uses (Djearmane et al., 2022).

2.3. Clay

Clay is a popular substrate for immobilizing various types of nanoparticles due to its numerous advantages over other fillers. It is cost-effective, safe, flexible, eco-friendly, and widely available. Additionally, clay boasts a high specific surface area, excellent binding capacity, high chemical stability, and ion exchange properties, as well as remarkable swelling adsorption. These features make clay an ideal material for designing and preparing biocompatible nanocomposites (Abdullah et al., 2019). Clay has been used by humans for thousands of years for a variety of purposes, including pottery, construction, and as a healing agent. Its unique properties, such as plasticity and low permeability, make it useful in many different applications. For example, it is commonly used in the manufacturing of ceramics, bricks, and tiles, as well as in the construction of earthen dams and other structures. Several types of clay have been used as a solid and stable support for synthesizing metal oxide/clay nanocomposites. These include bentonite (Aguiar et al., 2020); kaolinite (Chen et al., 2020), and zeolite (Roshanfekar Rad & Anbia, 2021). Clay minerals are primarily complex silicates that contain various ions such as aluminum, zinc oxide, and iron oxide.

2.3.1. Bentonite Clay

Bentonite clay is a type of absorbent aluminum phyllosilicate clay that has been used for various health and medicinal purpose since ancient times. It was named after Fort –Benton, Wyoming where it was first discovered in large quantities. It is also known as Montmorillonite after the French town of Montmorillonite where it was originally found many cultures around the world have consumed or applied bentonite clay for its therapeutic benefits (Mohammed, 2022). Bentonite unique properties make it a valuable material in various industries and applications. Its ability to absorb and adsorb impurities, as well as its swelling and thixotropic properties, makes it a versatile material with a wide range of uses.

Bentonite, a type of smectite clay, is a remarkable material with unique properties that make it an ideal raw material for a wide range of applications. Its 2:1 structure consists of one octahedrally coordinated and two silica sheets that are held together by van der Waals forces. Through isomorphic substitution of Al^{3+} , Si^{4+} , Mg^{2+} , and Fe^{3+} , bentonite contains a net of negative surface charge, which offers antifungal and antibacterial properties by generating superoxide radicals (Khansili & Murali Krishna, 2021). Bentonite also exhibits impressive colloidal behavior when it comes into contact with water, multiplying its volume multiple times to form a gelatinous suspension. This feature makes it incredibly valuable in diverse applications (Chen et al., 2020). This feature makes BT a valuable raw material for many diverse applications (Lambert, n.d.).

There are two main types of bentonite: calcium-type (Ca-BT) and sodium-type (Na-BT). Na-BT has a high capacity to adsorb water, causing it to expand many times its initial volume and form thixotropic suspensions in water. This property makes it useful in cement composites due to its swelling ability, high viscosity, and cation exchange capacity. Na-BT also possesses powerful negative ions that bind to positively charged ions in our bodies, offering increased protection against potential free radical cell damage (Khalifa et al., 2020). On the other hand, Ca-BT does not exhibit swelling behavior and therefore does not form thixotropic suspensions. Bentonite is composed mainly of montmorillonite, with other associated minerals such as feldspar, calcite, and quartz. The main component of dry BT is montmorillonite, which is built up by layers approximately 1 nm thick, each consisting of two tetrahedral and one octahedral unit (Al-Hammood et al., 2021a).

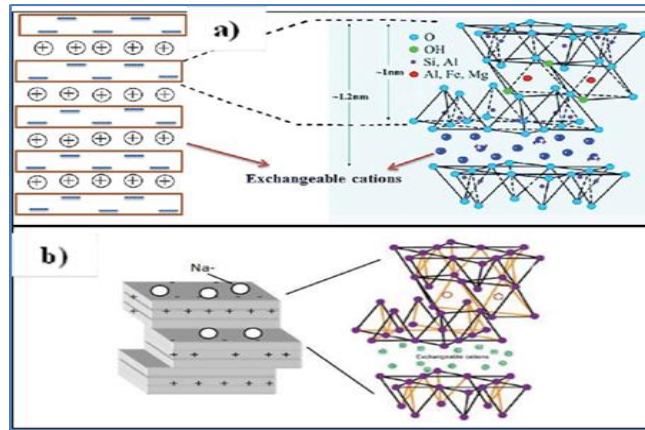


Figure 1. Diagrammatic sketch of the structure of bentonite clay (Hebbar et al., 2014). (a) and crystalline structure of sodium bentonite with inter lamellar water layer (b) (Zaher MS et al., 2018).

2.3.1.1. Properties of sodium bentonite

2.3.1.1.1 Physical properties

Na-BT is a type of bentonite clay that contains sodium ions in the interlayer space between its clay sheets. This unique property makes it incredibly useful for a variety of applications, including its ability to absorb large quantities of water, expand to several times its original volume, and form a gel-like mass. These features make it a valuable material for closing dams, drilling muds, water softening, and liquid clarification and Na-BT is a type of bentonite clay that contains sodium ions in the interlayer space between its clay sheets. This unique property makes it incredibly useful for a variety of applications, including its ability to absorb large quantities of water, expand to several times its original volume, and form a gel-like mass. These features make it a valuable material for closing dams, drilling muds, water softening, and liquid clarification (Park et al., 2016).

The physical properties of sodium bentonite are affected by several factors, including particle size, purity, moisture content, and pH. The particles of Na-BT typically range in size from 50 to 150 nm, with some particles as small as 1 to 2 nm. Its specific gravity is around 2.6, and the quantity that remains on a 200-mesh sieve should be less than 3% to ensure its quality. It is also important for sodium bentonite to have a moisture level of less than 10%, and its pH range is typically between 8 to 10.5 (Tagiev et al., 2022). Overall, Na-BT is a versatile material with

unique properties that make it incredibly useful in a variety of applications. Its ability to absorb water, expand, and form gels, as well as its high surface area and reactivity, make it an excellent adsorbent and catalyst. Its strong plasticity and bonding ability also make it ideal for molding and shaping materials.

2.3.1.1.2 Cation exchange capacity of bentonite clays

Bentonite Clays have a special ability to attract and hold positively charged ions, also known as cations, on their surface. This ability is known as their cation exchange capacity (CEC). The CEC of bentonite Clays depends on several factors such as their mineral composition, structure, and surface properties. The main component of bentonite clays is montmorillonite, which has a negative charge on its layers and edges. To balance this negative charge, exchangeable cations such as sodium, calcium, magnesium, and potassium occupy the interlayer spaces and external surfaces of the clay particles. The CEC of bentonite clays can vary from 50 to 150 milliequivalents per 100 grams of clay, depending on the type and number of exchangeable cations. This property is essential for many applications, including adsorption, catalysis, water treatment, and soil improvement (Lagashetty, 2019).

According to Rihayat et al.(2018) the higher the CEC, the higher the adsorption capacity and catalytic activity of the bentonite clay. Several factors can affect the CEC of bentonite clays. Increasing the pH can increase the CEC by increasing the negative charge on the clay edges while increasing the temperature can decrease the CEC by reducing the hydration of the interlayer cations. Increasing the salinity can also decrease the CEC by competing with the exchangeable cations for the clay surface sites. Acid activation, on the other hand, can increase the CEC by removing some of the interlayer cations and creating more negative sites on the clay surface. Therefore, the cation exchange capacity of bentonite clays is a crucial parameter that determines their ability to attract and hold positively charged ions.

2.3.1.1.3 Swelling properties

Swelling in clay particles occurs due to an increase in interlayer spacing. This happens when the clay is exposed to aqueous solutions with a brine concentration below a critical salt concentration. Bentonite is a widely used clay backfill material due to its low permeability and high swelling capacity, which are properties that stem from its content of montmorillonite, a clay

mineral with a negative charge that consists of two silica layers and one alumina layer (Siyao et al., 2022). The negative charge is balanced by positive ions, such as sodium, calcium, magnesium, potassium, etc., that are attached to the clay layers and surfaces. The quantity and type of cations, as well as the pH, temperature, salinity, and acid treatment of the clay, affect the amount it can swell. Na-bentonite, which contains more sodium cations, has a higher swelling capacity than Ca-bentonite and can increase its volume by up to eight times when wet, while calcium bentonite can only increase its volume by up to three times. Na-bentonite also has an increased number of smaller pores compared to Ca-bentonite, indicating its higher hydration and swelling capacity (Harrington et al., 2020).

Therefore Na-bentonite ability to swell is due to four primary mechanisms. Firstly, water molecules align themselves around the negatively charged surfaces of the clay layers, increasing interlayer distance and causing swelling. Secondly, oxygen atoms in the tetrahedral sheets interact with water molecules, enhancing hydration and creating excess electrons on the bentonite surface. Thirdly, the negative charge on the surface, resulting from the isomorphic substitution of aluminum by silicon, attracts water molecules and can be balanced by exchangeable cations like sodium (Adjei et al., 2021). Finally, sodium ions' smaller hydration radius and lower valence allow them to fit more easily between the clay layers and be displaced by water molecules, contributing to increased swelling capacity (Qin et al., 2021).

2.3.2. Application of bentonite clay

Bentonite clay is an incredibly versatile material with a wide range of applications in many different fields. It can be used as a filler, pellet, drilling mud, catalyst, and adsorbent, and has uses in structural polymers, ceramic bodies, adsorption catalytic processes, animal feed additives, wastewater treatments, and biomedical applications. Additionally, it can be transformed into high-value products, such as nanomaterials, medicines, and chemicals (Rihayat et al., 2018).

2.3.2.1. Bentonite as an antibacterial and antifungal agent

Bentonite clay is a fascinating substance with a wide range of applications, including its use for healing purposes. The unique healing properties of bentonite are derived from its crystalline structure, particularly the smectite group, which is composed of two octahedral alumina sheets localized between two tetrahedral silica sheets. This structure allows bentonite clay to intercalate

cationic bioactive agents and undergo interaction with various toxic species, exchanging them for essential minerals such as Fe^{3+} , Cu^{2+} , Al^{3+} , Ca^{2+} , or Na^+ (Zewudie et al., 2023). Furthermore, ability to exchange cations and provide essential minerals, bentonite clay also exhibits antibacterial and antifungal activity. This is due to its ability to adsorb and immobilize bacteria and fungi on its surface, alter the pH and water activity of the medium, create unfavorable conditions for microbial growth, and enhance the antimicrobial activity of other substances like zinc oxide, silver nanoparticles, and titanium dioxide. Bentonite clay's ability to combat bacteria and fungi is not only due to its antibacterial and antifungal properties but also its unique ability to interact with bacterial cells. When applied, the clay can physically damage bacterial cell membranes and walls, leading to the leakage of cellular contents and ultimately resulting in loss of viability. Moreover, bentonite clay can adsorb DNA molecules on its surface, preventing their chemical or enzymatic degradation. By blocking access to DNA lesions or inducing oxidative stress, it can also interfere with DNA repair mechanisms. These properties make bentonite clay an effective agent against harmful bacteria and fungi, as it not only prevents their growth (Z et al., 2020).

2.3.2.2. Bentonite as supporting materials for nanoparticles and nanocomposites

Sodium bentonite is a versatile type of clay that finds various applications as a supporting material for nanoparticles and nanocomposites. Its large surface area, swelling behavior, and high adsorption and ion exchange properties make it an excellent filler, binder, intercalation, supporting material, and substrate for metal/metal oxide nanoparticles. Bentonite has several advantages, including high cation exchange capacity, large specific surface area, low cost, and environmental friendliness (Sharma et al., 2023). Bentonite clay can enhance the stability, adsorption, and antibacterial properties of metal and metal oxide nanoparticles, such as silver, zinc oxide, titanium dioxide, and iron oxide. To improve its performance as a NCs material, bentonite can be modified using different methods such as ball milling, Solvothermal, and surfactant addition. The adsorption capacities of bentonite attract contaminants into the open pores, which increases the efficiency of dispersed transition metal oxides nanoparticles such as titanium oxide (TiO_2), zinc oxide (ZnO), zirconium oxide (ZrO_2), tin oxide (SnO_2), manganese oxide (MnO_2) and catalytic activity of these oxides is reinforced when they are supported on bentonite clay, which has a high specific surface area and low cost. The large number of ion

exchange sites available in clays is also beneficial for the incorporation of electro-active species and nanoparticles (Science, 2022).

2.3.3. Bentonite clay in Ethiopia

Bentonite clay resources are found in Ethiopia in Afar, and Southern, Nations, Nationalities, and People's Regional States. The Afar Regional State bentonite occurrences are easily accessible, as they are located near the main road. Most occurrences in Afar are located at Warssiso, Hadar, Gewane, and Ledi. The Warssiso (Mille) bentonite deposit is located 500 Km from Addis Ababa to Semera main roads. Gewane is located 376 Km northeast of Addis Ababa to Semera main road, Gewane bentonite deposit on 15 Km Gewane town. The Previous analysis of Warssiso bentonite indicates it is calcium bentonite (Zewudie et al., 2023).

2.4. Nanocomposite

Composites are solid materials created by combining two or more different materials, each with its own unique physical or chemical properties. These results in a new substance with enhanced properties compared to the original materials, in a specific finished structure. Composites are divided into three basic classes based on the size of the reinforcement in the structures: macro composites, micro composites, and NCs, which provide outstanding features and can be created by adding reinforcement below 100 nm in size. Due to their high surface-to-volume ratio, the interaction between the matrix and reinforcement is very high (Rangasamy, 2011). NCs have been frequently used in many applications, including engineering, plastic, rubber, coating, adhesive, electronic, and optic materials. To address the environmental concerns associated with the late decomposition periods of NCs in nature, NCs components are now being prepared from bio-renewable resources, such as vegetable oils, cellulose, starch, lignin, chitin, natural rubber, and proteins (Sabaruddin et al., 2021).

NCs offer several advantages over other composite materials, including a high surface/volume ratio that allows for small filler size and distance between fillers, better mechanical properties, improved optical properties, and scratch resistance. However, there are also some disadvantages, including concerns about toughness and impact performance, insufficient understanding of formulation/property/structure relationships, and a need for simpler particle exfoliation and dispersion (Ates et al., 2020; Omanovi et al., 2020). Iron oxide nanoparticles, especially α -Fe₂O₃, have gained attention for their use in medicine for targeted drug delivery, magnetic

resonance imaging, and as possible anticancer agents (Vihodceva et al., 2021). However, the low reactivity, agglomeration, and oxidation of iron oxide NPs can be avoided by coating them with another metallic oxide such as ZnO NPs.

The combination of ZnO and α -Fe₂O₃ NC has proven to be a promising antibacterial and antifungal material that is biocompatible and displays low toxicity. The surface coating of iron oxide NPs not only decreases their cytotoxicity but also increases their stability and efficiency in antimicrobial potential. The antibacterial effect of the surface coating may depend on other factors such as the concentration, morphology, and solubility of the nanoparticles. Green synthesis of iron oxide NPs and their coating by ZnO NPs in ambient temperature can increase the reactivity of iron oxide NPs as antimicrobial and prevent their agglomeration and oxidation (Abbas et al., 2020b).

2.4.1. Hematite /bentonite

To use nanoparticles as antibacterial agents, they must be applied to supporting substrates such as graphite, zeolite, carbon nanotube, clays, or polymer materials. Clays, such as bentonite, montmorillonite, kaolinite, halloysites, and palygorskite, are commonly used supporting substrates for NPs due to their excellent and expandable properties. Hematite nanocrystals have recently gained scientific attention for antibacterial applications, and they have been approved by the FDA for food and medical applications (Dutta & Wang, 2019; Krishnan & Mahalingam, 2017b) and bentonite shares several characteristics with hematite, including antimicrobial potency, active site type, high adsorption capacity, ionic exchange capacity, surface acidity, large surface area, and thermal stability.

Bentonite is a naturally sourced and abundant material that is cheap, eco-friendly, stable, and a selective catalyst. Its use as a supporting substrate for hematite nanoparticles offers several advantages over other supports. Moreover, bentonite can be used to develop new materials with a micro-porous structure involving new catalytic sites by cation incorporation. The combination of hematite and bentonite can result in a stable and biocompatible material that displays low toxicity and has enhanced antimicrobial properties. This NCs material has potential applications in various fields, including water treatment, biomedical applications, and food packaging. Its broad range of advantages makes it an attractive option for researchers looking to develop new materials for antibacterial applications (Campos et al., 2015; A. Kumar & Lingfa, 2020).

2.4.2. Zinc oxide / Bentonite nanocomposite

There are challenges in the large-scale utilization of nanoparticles such as ZnO in water treatment due to difficulty in their separation and recovery after treatment. In addition, the use of metal oxides nanoparticles for water treatment has some disadvantages, namely: (1) higher colloidal stability in aqueous solution, (2) agglomeration of the nanomaterials at high concentrations, and (3) difficulty in separating and recovering the nanomaterials after use (Lei et al., 2017). However, steps have been adopted to overcome these shortcomings. These include doping and co-doping of metal oxide nanomaterials and immobilization of nanomaterials on suitable matrices (Darvishi Cheshmeh Soltani et al., 2016). This suitable substrate could function as support to overcome the difficulties involved in post-separation and recovery. Moreover, the support of nanosized semiconductor materials on matrices could help to enhance their activity when compared with ordinary nanomaterials

Clay matrices, such as kaolinite, montmorillonite, and bentonite, have been successfully employed as support for nanosized ZnO NPs. Immobilizing and anchoring ZnO NPs on the surface of clay minerals provide more active surface sites, reduces agglomeration, and prevents the release of nanoparticles into the environment. Clay NCs have become major components in recent research findings for tackling environmental pollutants. ZnO NPs are cheap, non-toxic, and capable of removing emerging contaminants. Using NPs alone can be dangerous due to their size effects on human health and the environment. To overcome this problem, hybrid processes have been introduced by combining nanoparticles with other environmentally friendly materials, such as inert and stable materials, to utilize their full properties in wide-ranging applications. Moreover, introducing NPs with other substrates or fillers can result in composites with novel and enhanced properties that cannot be achieved by the individual components (Pouraboulghasem et al., 2016).

2.4.3. Synthesis and characterization of nanocomposites

To improve the performance and application of nanocomposites (NCs), they must be synthesized using approaches that lead to the assembly of consistently distributed particles and consistent morphologies. This will increase the homogeneity and limit of quantification. Some components also function best when NCs are used as building blocks, and a more uniform, simultaneous embedding over the NP's nanocrystalline matrix will result in better performance (Sen, n.d.). NPs

can be added to a material's nanostructured composite to maintain high strength while enhancing ductility and toughness. The two most widely used methods for the syntheses of NCs are top-down and bottom-up procedures. Top-down methods, such as mechanical grinding, high-energy ball milling, mechanical alloying, reactive milling, and lithography, use mechanical techniques. Bottom-up chemical techniques include hydrothermal, co-precipitation, micro-emulsion, sol-gel, chemical vapor deposition, electrodeposition, and colloidal dispersion (Pillai et al., 2020), as shown in Figure 2.

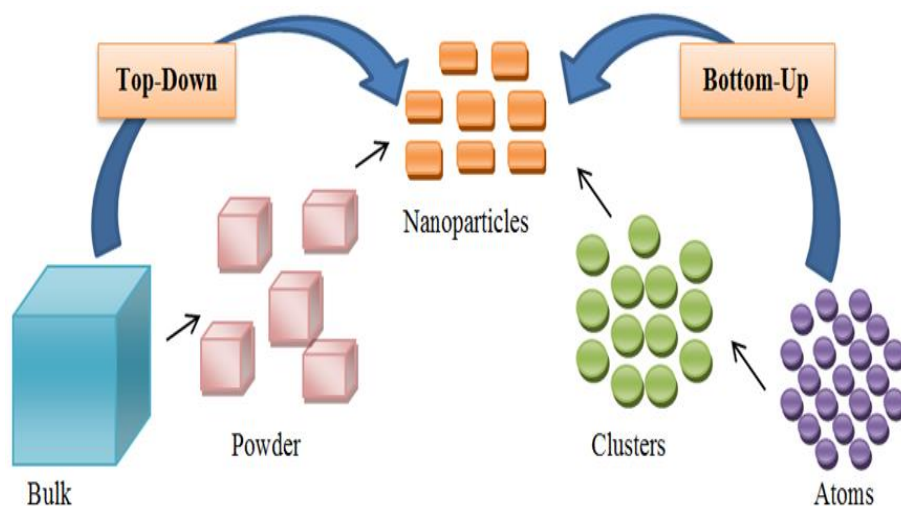


Figure 2.Top-down and bottom-up synthesis (Gregorczyk & Knez, 2016)

Various synthetic methods have been reported by researchers which describe the formation of NPs and can be divided into three classes such as physical, chemical, and biological. Physical and chemical methods are the most commonly used methods for synthesizing NPs. Physical methods include laser ablation, sputtering, and mechanical milling, while chemical methods include chemical reduction, co-precipitation, seeding, micro-emulsion, hydrothermal synthesis, and electron deposition. Biological methods include biosynthesis, bio-reduction, and bio-fabrication. Each method has its advantages and disadvantages, and the choice of method depends on the desired properties of the NPs and the intended application (Rangasamy, 2011). Different methods of synthesis of NPs are summarized in Figure 3 below.

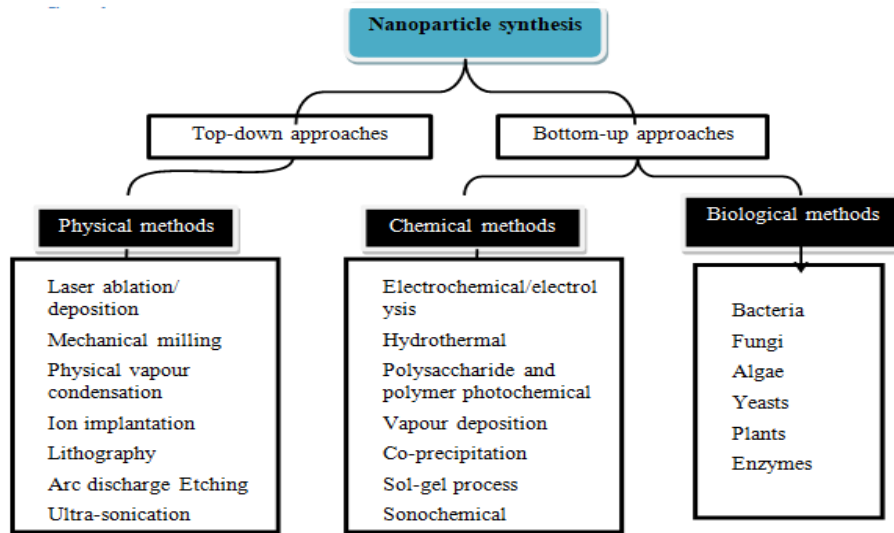


Figure 3. Different methods for the synthesis of NPs (Ganta et al., 2022).

2.4.3.1. Biological synthesis

The biological synthesis of nanoparticles is a promising green approach that combines nanotechnology and biotechnology. However, biological nanoparticles are not monodispersed and are synthesized slowly due to the varying concentration of macromolecules and nucleation components, resulting in polydispersity and reduced synthesis rates (Bandeira et al., 2020; Jadoun et al., 2021). To address these issues, optimization of microbial cultivation methods and extraction techniques, as well as a combinatorial approach like photo biological methods, may be necessary. Detailed study of cellular, biochemical, and molecular mechanisms involved in biological NPs synthesis is crucial for improving their properties and increasing the synthesis rate. The potential for using plants and microbes as biological materials for NPs synthesis remains largely unexplored. Characterization of polymer NCs is essential for understanding their properties. The quality of filler dispersion, surface modification effects, and interactions between filler modification and polymer chains must be analyzed. Changes in process parameters and a wide spectrum of properties must also be considered (Ganta et al., 2022).

2.4.3.2. *Hagenia abyssinica*

Hagenia abyssinica (*H. abyssinica*), commonly known as Kosso in Ethiopia, is a plant species that has garnered significant attention for its potential use in the synthesis of biological nanoparticles. Specifically, researchers have focused on the use of *H. abyssinica* seed extract for the synthesis of metal oxide nanoparticles. These nanoparticles have been of interest due to their unique properties and potential applications in various fields, including medicine, electronics, and catalysis. *H. abyssinica* seed extract has been found to contain various secondary metabolites, including phenolic compounds, alkaloids, and flavonoids, which act as reducing agents in the synthesis of metal oxide nanoparticles. These metabolites can facilitate the reduction of metal ions to form nanoparticles with a narrow size distribution and unique properties (Zewudie et al., 2023).

Furthermore, *H. abyssinica* has been found to possess antibacterial and antifungal properties due to its secondary metabolites. The use of *H. abyssinica* seed extract as a reducing agent in the synthesis of metal oxide nanoparticles has the potential to produce NPs with antibacterial and antifungal properties, making them useful in various biomedical applications (Murthy, et al., 2020). When producing nanoparticles on a large scale, it's common to use *H. abyssinica* as a capping agent. However, if left uncapped, these particles can become hazardous to the environment due to their larger size and tendency to aggregate. Therefore, it's important to choose appropriate capping agents that can stabilize colloidal solutions and facilitate uptake into living cells and the environment (Agidew, 2022).

Capping agents play a crucial role in preventing overgrowth and aggregation of nanoparticles during colloidal synthesis. They stabilize the interface between the nanoparticles and their preparation medium, altering the surface chemistry and size distribution of the particles. To be effective, capping agents should be biodegradable, well-dispersed, biocompatible, and non-toxic. This reduces their non-specific interaction with biological components, resulting in lower cellular toxicity. The specific structural features of nanoparticles are attributed to capping on their surface, and the choice of capping ligands can significantly impact their biological activities and environmental impact. Therefore, it's essential to carefully consider the selection of capping agents for nanoparticle synthesis (Javed et al., 2020).



Figure 4. Hagenia abyssinica plant

2.4.4. Applications of α -Fe₂O₃ and ZnO nanocomposites

The α -Fe₂O₃ NPs chemical, physical, and biological properties have increased their commercial and research applications in the pharmaceutical, food, cosmetic, environmental, and antibacterial and antifungal application industries (El-Maghrabi et al., 2021). The current application of α -Fe₂O₃ NPs is of great importance due to their industrial application (Nasrollahzadeh et al., 2019). At present, multiple studies have been published that show the antimicrobial effect of α -Fe₂O₃ NPs against Gram-negative and Gram-positive bacteria and fungi (Armijo et al., 2020). In cosmetics industries, especially for the production of lotion, Fe₂O₃ NPs are used for healing (Schalka et al., 2019). Fe₂O₃ in α -Fe₂O₃ is an inorganic compound that is often added to sunscreen creams due to its reddish color and potential as an ultraviolet (UV) absorber for instance, showed that water-oil emulsions containing α -Fe₂O₃ can provide effective sun protection for both ultraviolet A (UVA) and ultraviolet B (UVB) ranges (Bernstein et al., 2021). ZnO can be used in a wide range of sectors as well (Kolodziejczak-Radzimska & Jesionowski, 2014). In addition to being utilized as a skin protectant and sunscreen in cosmetic applications, it serves as a UV safety net to guard against aging caused by ultraviolet rays (Pinnell et al., 2000).

2.4.4.1. Antibacterial activity

Antibacterial activity is a crucial property for materials used in various applications such as medicine and food packaging. Bacteria can be classified into Gram-positive and Gram-negative based on their cell wall structures, and Antibacterial activity can be measured using the activity index (AI) obtained by comparing the inhibition zone of the test sample to that of the positive control. The zone of inhibition is the area where no bacterial growth is observed due to the antibacterial action of the test nanocrystals suspended in an appropriate solvent such as dimethyl Sulfoxide. Standard antibiotics such as ampicillin and gentamicin are commonly used as positive controls (Weldegebräel, 2020).

Nanocomposites have been extensively studied for their antibacterial properties, but some have limitations that require further investigation. For instance, Ag/TiO₂/bentonite NCs have shown enhanced antibacterial activity against both Gram-positive and Gram-negative bacteria, but they exhibited a nontoxic effect only at low concentrations against HEK 293 cells. On the other hand, both ZnO and α -Fe₂O₃ NPs possess antibacterial activity, but ZnO nanoparticles have higher toxicity than α -Fe₂O₃ NPs, despite their higher antibacterial activity. Therefore, α -Fe₂O₃/ZnO (Abbas et al., 2020a) NCs have been synthesized using various methods such as hydrothermal, photo-reduction, and microwave-assisted synthesis, and have been found to exhibit higher antibacterial activity than ZnO NPs (Vihodceva et al., 2021).

The α -Fe₂O₃ NPs are a type of magnetic NPs that has various applications in bio-nanotechnology, such as drug delivery, contrast enhancement, hyperthermia, and antimicrobial activities. They have several advantages, including bio-compatibility, chemical stability, and low toxicity. One eco-friendly and safe method to synthesize α -Fe₂O₃ NPs is green synthesis, which utilizes plant extracts as reducing agents. Some studies have reported the antibacterial efficacy of α -Fe₂O₃ NPs against different bacteria, such as *S. aureus* and *E. coli*. The possible mechanisms of antibacterial action include the production of reactive oxygen species (ROS), membrane damage, oxidative stress, and DNA damage (Rodrigues et al., 2019).

Previous studies suggest that ZnO and α -Fe₂O₃ nanoparticles interact with bacteria and exhibit multiple antibacterial mechanisms. The NPs can strongly perturb the essential functions of cell membranes and directly adhere to bacteria due to their opposite charges. The NPs damage the cell wall and membrane structure, leading to cytoplasm leakage, inhibiting biofilm aggregation,

and reducing bacterial motility. Furthermore, ROS produced by the nanoparticles can permeate into cells and destroy DNA, resulting in bacterial death (da Trindade et al., 2020; Nazeer et al., 2018). Research has shown that the antibacterial activity of ZnO and α -Fe₂O₃ NPs against *S. aureus* and *E. coli* is due to the decay of bacterial outer membranes by ROS, primarily •OH, which leads to phospholipid peroxidation and ultimately cell death (Abbas et al., 2020a). Nanomaterials that physically attach to a cell membrane can be bactericidal if they come into contact with the bacterial cell. If the membrane of a bacterium is conceded, the cell could repair itself or, if the cut is severe, the cell component may release, and the cell will eventually die (Saied et al., 2022; Shahbazi, 2021).

2.4.4.2. Antifungal activity

ZnO nanoparticles have a high surface area-to-volume ratio, which greatly enhances their reactivity and antimicrobial properties. Their small size allows them to easily penetrate fungal cell walls, leading to increased disruption of cellular functions such as membrane permeabilization and oxidative stress induction. ZnO NPs can be functionalized with organic molecules to improve their solubility and stability, resulting in heightened surface reactivity and effective antibacterial action at low concentrations (Sun et al., 2018). Flower-shaped ZnO NPs have been found to exhibit antimicrobial effects against both gram-positive and gram-negative bacteria (R. Kumar et al., 2017). On the other hand, α -Fe₂O₃ NPs have been shown to possess antifungal activity by generating reactive oxygen species (ROS) that induce oxidative stress in fungal cells, leading to cell death. The small size of α -Fe₂O₃ NPs also enables them to penetrate the fungal cell wall, enhancing their antifungal activity.

The α -Fe₂O₃ NPs can be functionalized with organic molecules to improve their biocompatibility and target-specific delivery. ZnO and α -Fe₂O₃ NPs have been reported to exhibit antifungal activity against various fungal pathogens (Abbas et al., 2020a). ZnO NPs disrupt fungal membranes and release Zn⁺² ions, which interfere with fungal metabolism and growth, while α -Fe₂O₃ NPs exhibit antifungal activity through their catalytic activity, generating hydroxyl radicals that damage fungal cells. Combining ZnO and α -Fe₂O₃ NPs to form NCs improves their stability, dispersibility, and biocompatibility, enhancing their antifungal activity. Bentonite-based NCs have also been found to inhibit the growth of fungi (Krishnan & Mahalingam, 2017a).

For one group of fungal strains, *Candida albicans*, NPs, and NCs prepared from different precursors demonstrated the best antifungal activity. While confirmed mechanisms for destroying microbes by α -Fe₂O₃ and ZnO NPs were not found in existing literature, it is believed that electrostatic attraction between metal ions and the surface of cell walls damages cell integrity, allowing entry into the inner part of cells followed by the production of reactive oxygen species (ROS) (Skłodowski et al., 2023). These ROS molecules are responsible for damaging DNA, denaturing proteins, rupturing enzymes, and depleting antioxidant glutathione levels, ultimately leading to cell death as indicated in Figure 5. Combining ZnO and α -Fe₂O₃ NPs to form NCs improves their stability, dispersibility, and biocompatibility, enhancing their antifungal activities

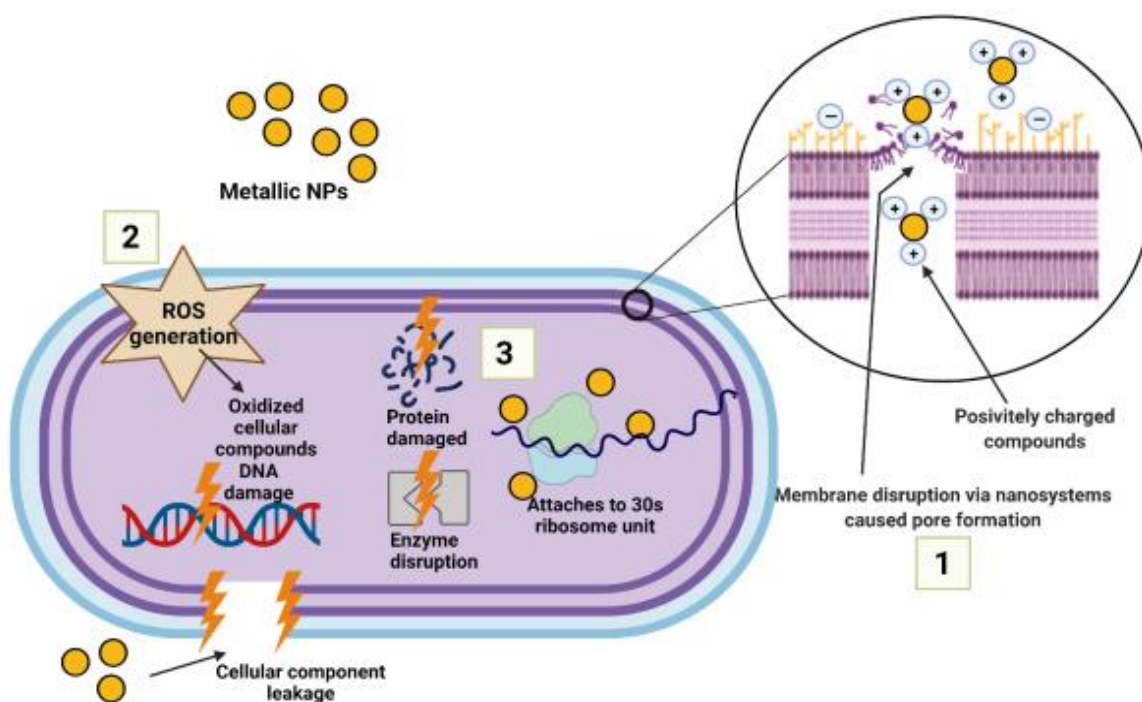


Figure 5. The main mechanisms of antimicrobial activity of metal nanoparticles include: (1) disruption of the pathogen cell wall resulting in increased permeability, (2) generation of ROS disrupting redox homeostasis and damaging cellular structures, (3) binding to intracellular structures causing their damaging.

CHAPTER 3

MATERIALS AND METHODS

3.1. Description of sample collection site

Raw Bentonite was collected from Mille (Warssiso) (Elevation of 518 m above sea level, weather: 27 °C, Wind: W at 4.45 km/h, 46 % Humidity, Latitude: 11° 25' 18" N, Longitude: 40° 45' 46" E. 500 km from Addis Ababa), woreda of Arsi Rasu (Administrative Zone 1), located in Afar Region, north-eastern Ethiopia, while *H. Abyssinica* was collected from East Arsi Zone.

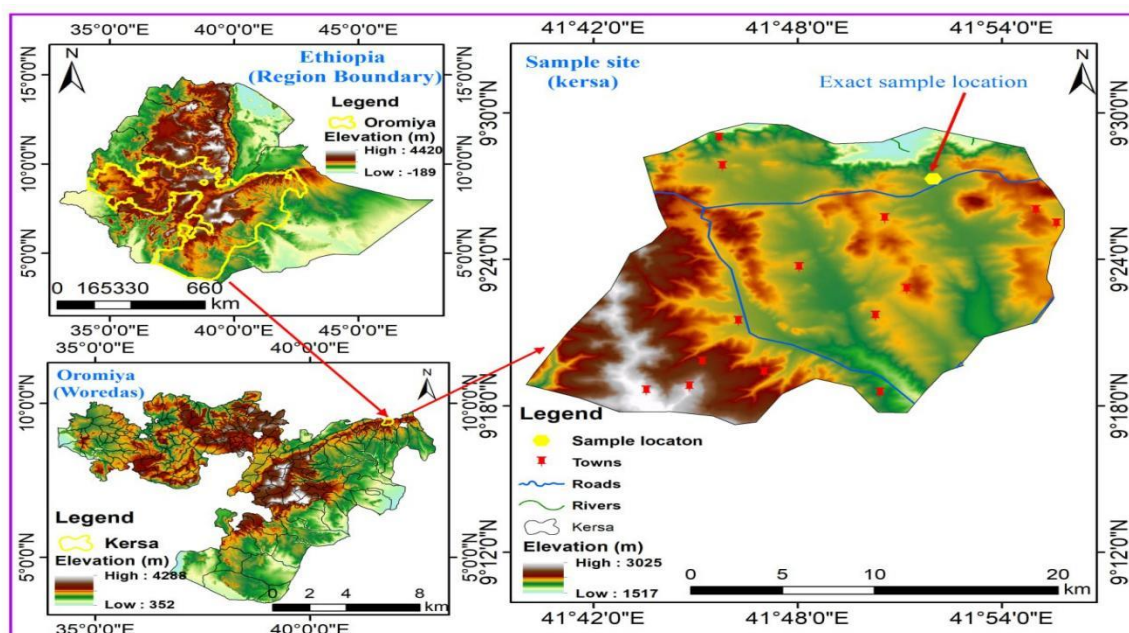


Figure 6. Location map of *H. abyssinica* sample collection site.

3.2. Research site

Synthesis of ZnO/bentonite, Fe₂O₃/bentonite, and ZnO/Fe₂O₃/bentonite NCs was carried out in the Applied Chemistry Laboratory of Adama Science and Technology University (ASTU). UV-Vis DRS, PL, TGA, and XRD analysis were also performed in ASTU. FTIR measurements were done at Bahir Dar University. SEM characterization was done in South Korea and Hindi respectively. The bacterial strains, Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria used, and fungi strain, candida albicans for checking antibacterial and antifungal activity were taken from American Type Culture Collection (ATCC) found in Adama Public Health Research and Referral Hospital Laboratory Center.

3.3. Materials

3.3.1. Chemicals and Reagents

All the chemicals used in this study were analytical grade these are ferric chloride hexa hydrate (Sigma-Aldrich, 99% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Hydrochloride acid (HCl, Riedel dehaen, Germany, 37% HCl), Sodium hydroxide (Sigma- Aldrich 99% NaOH), Na_2CO_3 (98%), Ethanol (99.8%, Fmd, Ethiopia), $\text{ZnNO}_3 \cdot 6\text{H}_2\text{O}$ (98%) and were used as received without further.

3.3.2. Apparatus and instruments

The apparatus and equipment that were used during this research work were Uv-Vis Diffuse reflectance spectroscopy (Uv-Vis DRS Elico SL 150 spectrophotometer), XRD (Shimadzu, XRD7000S), FTIR (FTIR Perkin Elmer's spectrophotometer), PL (Photoluminescence spectroscopy), SEM (Scanning Electron Microscopy), CEC(Cation Exchange Capacity), (TGA (Thermo gravimetric Analysis), electronic balance, oven, beaker, test tube, magnetic stirrer, thermometer, What man No.1, filter paper, measuring cylinder, pH-meter, spatula, Erlenmeyer flask, volumetric flask, ceramic crucible, Buchner funnel, and bar magnet.

3.4. Experimental method

3.4.1. Purification of bentonite

The natural bentonite sample collected from Afar was air-dried and crushed to a particle size using a mortar and pestle. To eliminate impurities such as quartz carbonates, calcites, and iron-hydroxide, the crushed bentonite was dispersed in distilled water (100 g/L) at room temperature. The clay fraction was then recovered using a homogenizer (1000 rpm) for 1 hour. This process was repeated four times to obtain uniform suspension. The suspensions were allowed to settle for 24 hours to remove any impurities. The supernatant was decanted and any remaining sediment in the flask was discarded. The suspension was centrifuged at 3000 rpm for 20 minutes, dried in an oven at 60°C, ground, sieved through a 63 μm mesh, and stored in tightly closed plastic bottles for characterization and use in experiments (Gong et al., 2016).

3.4.2. Activation of bentonite clay by sodium carbonate (Na_2CO_3)

The activation of the purified bentonite was performed using a modified version of the procedure. First, 100 g of purified bentonite powder was dispersed in 1 L of distilled water to form a suspension. Then, 5 g of $\text{Na}_2\text{CO}_3 \cdot 10 \text{H}_2\text{O}$ was added to the suspension to obtain a stable aqueous dispersion. The suspension was stirred slowly and boiled for 1 hour. After boiling, the suspension was allowed to cool to room temperature and diluted with 1 L of distilled water, and allowed to stand for 24 hours. The supernatant was decanted and any bottoms settled in the flask were discarded. The Na-bentonite suspension was centrifuged at 3000 rpm for 20 minutes then dried until dry for several days and ground to obtain activated bentonite powder for further experiments (Zewudie et al., 2023).

3.4.3. Collection of *Hagenia Abyssinica* plant leaves and preparation of its powder

The fresh leaves of the *H. abyssinica* collected from west Arsi zone were washed three times with tap water followed by distilled water to remove extraneous matter. The cleaned leaves were then completely dried at room temperature in an open air and under shadow for about two weeks to remove any remaining moisture content from the leaves. The dried leaves were crushed by using an electrical grinding machine to produce a fine powder, followed by packing in a brown bottle to protect against the effect of light (Murthy, Desalegn, et al., 2020).



Figure 7. Steps involved in the preparation of *H. abyssinica* plant powder

3.4.4. Preparation of aqueous plant leaf extract

To prepare plant leaves to extract, 25 g of powdered *Hagenia abyssinica* leaves were added to a 1000 mL conical flask containing 400 mL of distilled water. The flask was covered with aluminum foil to prevent exposure to light. The whole content was heated at 50°C on hot plate while stirring with magnetic stirrer for 30 minutes. Then the solution was left to cool overnight at room temperature. The resulting solution was filtered through filter paper to obtain a pure liquid, which was stored at 4 °C for further use (Murthy, Zeleke, et al., 2020).

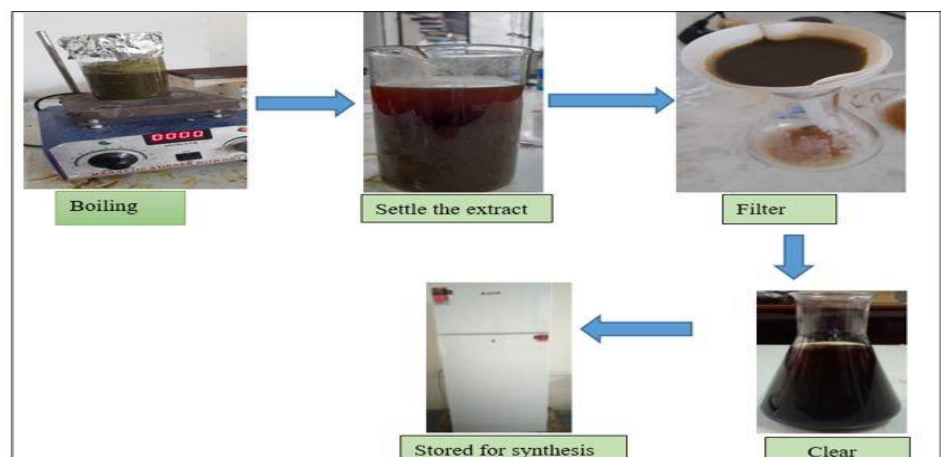


Figure 8. Show preparation steps of aqueous plant leaves extract.

3.4.5. Phytochemical screening

The extracts from *H. abyssinica* leaves were screened for the presence of naturally occurring biologically active compounds such as alkaloids, carbohydrates, phenols, saponins, flavonoids, steroids, and glycosides. The screening was carried out as per the standard methods (Zewudie et al., 2023).

3.4.5.1. Test for saponins

Deionized water (5 mL) was added to the crude extract in a test tube. The mixture was shaken vigorously for 2 minutes. Persistent foaming on shaking indicated the presence of saponins (Ali & Endalew, 2021).

3.4.5.2. Test for alkaloids

The crude extract (3 mL) was mixed with 1% HCl (2 mL) and then heated for 20 minutes in a steam bath. The mixture was filtered after cooling. A few drops of Wagner 's reagent was added.

The existence of alkaloids was indicated by the turbidity of the resulting precipitate (Sapunyo et al., 2023).

3.4.5.3. Test for phenols

Aqueous leave extract was dissolved in 5 mL of ethanol mixture and added 2 drops of 5% FeCl₃ solution were added. A greenish precipitate showed the presence of phenols (Zewudie et al., 2023).

3.4.5.4. Test for steroids

Mixed chloroform (2 mL) with the crude extract (2 mL), and then by adding 2 mL each of concentrated H₂SO₄ and acetic acid (poured into the mixture separately). The green color confirmed the presence of steroids (Razmavar et al., 2014).

3.4.5.5. Test for flavonoids

To 2 mL extract, 2 mL of 2% NaOH solution was added. A deep yellowish color which turned colorless when a few drops of diluted acid were added showed the presence of flavonoids (Al-khazraji, 2018).

3.4.5.6. Test for glycosides

The crude extract (1 mL) was mixed with chloroform (2 mL). Then, 2 mL of concentrated H₂SO₄ was carefully poured into the mixture and shaken gently. A reddish-brown color was formed which showed the presence of glycosides (Salkowski's test) (Al-khazraji, 2018).

3.4.5.7. Test for carbohydrate

A mixture of equal volumes of Fehling A and Fehling B reagents (2 mL each) was mixed with the crude extract (2 mL) and gently boiled. Brick red precipitate indicated the presence of reducing sugars (Fehling's test) (Agidew, 2022).

3.4.6. Synthesis of zinc oxide nanoparticles

The zinc oxide nanoparticles (ZnO NPs) were synthesized using different volumes ratios of 0.05 M zinc nitrate hexa hydrate solution with leaf extract prepared from 20 g in 400 ml distilled water. First 100 ml of plant leaf extract was mixed with 100 ml of 0.2 M zinc nitrate hexa hydrate solution (1:1), next 200 ml of 0.2 M zinc nitrate hexa hydrate solution, and 400 ml leaf extract solution (1:2), followed by adding drop wise 200 ml of 0.2 M zinc nitrate hexa hydrate

solution with constant stirring to 100 ml of leaf extract (2:1). Then the solution was continuously stirred for 3 hr at 80°C through adjusting the pH of the solution at 12 by 1 M of NaOH solution. Then, the solution was cooled and centrifuged at 3000 rpm for 20 min to separate the precipitates. The precipitates were washed several times with double distilled water and dried at room temperature. The Solid powders were ground using mortar and pestle and followed by calcination at 438.2°C for 2hr in a muffle furnace. The solid powders were then ground using a mortar and pestle and subjected to calcination at 438.2°C for 2 hours in a muffle furnace (Golmohammadi et al., 2020).

3.4.7. Preparations of Iron Oxide Nanoparticles (α -Fe₂O₃NPs)

The synthesis of iron oxide nanoparticles involved using different ratios of plant extract. To prepare the plant extract, 20 g of plant material was mixed with 300 ml of distilled water and boiled for 20 minutes. The resulting solution was then filtered to obtain the plant extract. For the synthesis of iron oxide nanoparticles, three different volume ratios of 0.2 M ferric chloride hexa hydrate solution and the plant extract were used. The first ratio was 1:1, where 100 ml of the plant extract was mixed with 100 mL of the ferric chloride hexa hydrate solution. The second ratio was 1:2, where 100 ml of the ferric chloride hexa hydrate solution was mixed with 200 mL of the plant extract after mixing the solution, a black, particle-filled solution was created and continuously stirred for three hours using a magnetic stirrer. To form a precipitate and adjust the pH of ferrous/*Hagenia abyssinica* mixed solution, 1M NaOH solution was added drop by drop until the pH reached 10-12, followed by stirring for 30 minutes. The resulting solid product was extracted using centrifugation and cleaned three to four times with distilled water before air drying. Finally, the dry product was calcined in a muffle furnace at 587.7°C for three hours, producing a reddish-brown powder (Hayet et al., 2021).

3.4.8. Preparations of iron oxide /bentonite Nanocomposites (α -Fe₂O₃/ Bentonite NCs)

The preparation of α -Fe₂O₃/bentonite nanocomposites involved using three different ratios of bentonite and ferric chloride hexa hydrate. To prepare the nanocomposites, 6g of bentonite was dispersed in 100 ml of distilled water with magnetic stirring for 30 minutes. Then, 0.2M ferric chloride hexa hydrate solution was added to the bentonite suspension with different ratios. The first ratio was 1:1, where 6 g of bentonite was mixed with 6 mL of ferric chloride hexa hydrate solution. The second ratio was 1:2, where 6g of bentonite was mixed with 3 mL of ferric chloride

hexa hydrate solution. The third ratio was 2:1, where 6 g of bentonite was mixed with 12 mL of ferric chloride hexa hydrate solution. *H. abyssinica* extract (100 mL) was then added to each beaker, and the solution was stirred for 2 hours at 80°C. The resulting mixture was stirred for 1 hour and then subjected to sonication for 10 minutes to obtain a uniform dispersion. After sonication, the mixture was heated at 80°C for 3 hours and the pH was adjusted to 12 using a 1 M NaOH solution. Extracted the solid using centrifugation and washed it three times with deionized water to remove any impurities. Then, the solid was dried at a temperature of 100°C until dried. Finally, the dried composite powders were calcined above 583.4°C for three hours in a muffle furnace to obtain the α -Fe₂O₃/Bentonite nanocomposite powder and stored in a sample holder for further activities (Orolínová & Mockovčíaková, 2009).

3.4.9. Preparation of ZnO/bentonite Nanocomposite

To prepare ZnO/bentonite nanocomposites, three different ratios of bentonite and zinc nitrate hexa hydrate were used. Firstly, 6g of bentonite was dispersed in 100 mL of distilled water with magnetic stirring for 30 minutes. Next, 0.2 M zinc nitrate hexa hydrate solution was added to the bentonite suspension with different ratios. The first ratio was 1:1, where 6g of bentonite was mixed with 6 mL of zinc nitrate hexa hydrate solution. The second ratio was 1:2, where 6 g of bentonite was mixed with 3 ml of zinc nitrate hexa hydrate solution. The third ratio was 2:1, where 6g of bentonite was mixed with 12 mL of zinc nitrate hexa hydrate solution. *H. abyssinica* extract (100 mL) was then added to each beaker, and the solution was stirred for 2 hours at 80°C. Afterward, the pH of the solution was adjusted to 12 using 1 M NaOH and stirred for an additional 30 minutes at the same temperature. The resulting light brown precipitates were centrifuged and washed with double distilled water and ethanol several times. The samples were then dried at room temperature, subjected to calcination at 646°C for 3 hours, and finally stored in a glass bottle until needed (Kassem, 2015).

3.4.10. Preparation of α -Fe₂O₃/ZnO/Bentonite Nanocomposites

The preparation of zinc oxide/iron oxide/bentonite nanocomposites involved using three different ratios of zinc oxide bentonite, and iron oxide. To prepare the nanocomposites, 4 g of bentonite was dispersed in 100 mL of distilled water with stirring for 30 minutes. Then, 0.15 M ferric chloride hexa hydrate solution and 0.15 M zinc nitrate hexa hydrate solution were added to the bentonite suspension with different ratios. The first ratio was 1:1:1, where 4 g of bentonite was

mixed with 4 mL of ferric chloride hexa hydrate solution and 4 mL of zinc nitrate hexahydrate solution. The second ratio was 1:1:2, where g of 4g bentonite was mixed with 2 mL of ferric chloride hexa hydrate solution and 2 mL of zinc nitrate hexa hydrate sol. The third ratio was 2:2:1, where 2g of bentonite was mixed with 4 mL of ferric chloride hexa hydrate solution and 4 mL of zinc nitrate hexa hydrate solution. The resulting mixture was stirred for 1 hrs and then subjected to sonication for 10 minutes to obtain a uniform dispersion. *H. abyssinica* extract (100 mL) was then added to each beaker, and the solution was stirred for 2 hours at 80°C. After stirring, the mixture was heated at the same temperature for 3 hours and then pH was adjusted to 12 using a 1 M NaOH solution. The solution was then cooled overnight and centrifuged at 3000 rpm for 20 minutes to separate the precipitates. The final precipitates were then collected and washed several times with distilled water and ethanol to remove impurities and the prepared material was calcined in a muffle furnace at 565.7°C for 3 hours (Figure9). The resulting powders were labeled as ZnO/ α -Fe₂O₃/Bentonite powder and collected for characterization (Kamali et al., 2022).

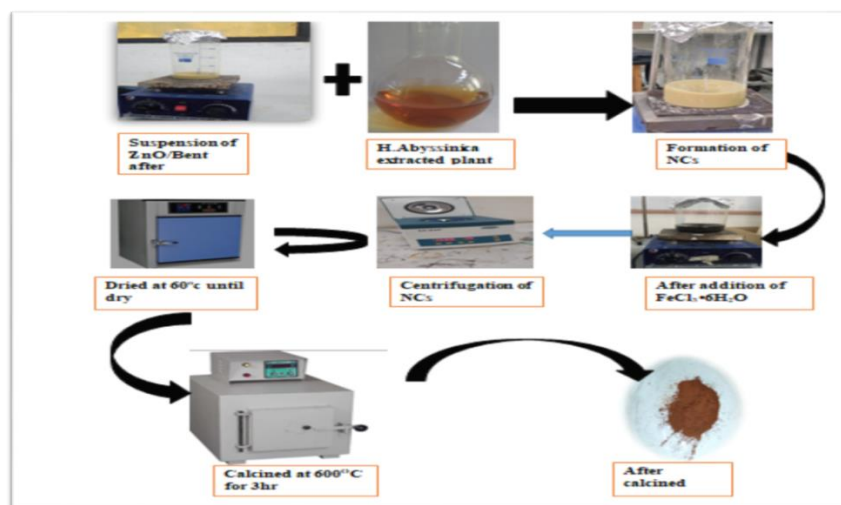


Figure 9. The schematic diagram of the synthesis procedure for α -Fe₂O₃/ZnO/Bentonite.

3.5. Characterization

Different characterization techniques were performed to investigate the crystalline structure, optical properties, and various phytochemical constituents involved in the reduction, and morphology of the synthesized NPs and NCs. The detailed procedures are mentioned below for each technique (Joudeh & Linke, 2022).

3.5.1. X-ray diffractometer

To determine crystallinity, and crystallite size of the synthesized α -Fe₂O₃, ZnO, bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃/Bentonite XRD were examined by using the Shimadzu XRD-700 X-ray diffract meter (XRD) at Adama Science and Technology University Material Science Department. The XRD patterns were recorded at the range of 2θ from 3 to 80° with a scan rate of 20/ min using Cu K α ($\lambda = 1.5406 \text{ \AA}$) radiation with an accelerating voltage of 40 KV. The crystallite size D of a particle can be estimated according to the Scherrer equation:

$$D = \frac{k\lambda}{\beta \cos \theta} \quad \text{Equation 1}$$

Here, the x-ray wavelength λ is 0.154 nm, k is the structure factor, which is assigned a value of 0.94, D is the average diameter of the crystals, θ is the Bragg angle in degrees, and β is the full-width at half-height of the prominent peaks (Mittal, 2012; Séby, 2021).

Sample Preparation: 1.5-2.5 grams of dried α -Fe₂O₃, ZnO, and bentonite, as well as ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃ /bentonite nanocomposites, were ground into a paste using a mortar and pestle. The resulting paste was then filled into a sample holder and subjected to X-ray diffraction analysis.

3.5.2. Scanning Electron Microscope

SEM analysis of the biosynthesized was carried out by using SEM (JCM-6000 plus BENCH TOP SEM, SHIMADZU Corporation) in South Korea, which reveals information about the external morphology of the biosynthesized α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite and ZnO/ α -Fe₂O₃ /Bentonite sample with direct visualization.

Sample Preparation: A small amount of each powder sample was coated with gold to make them conductive using a coater.

3.5.3. Fourier Transform Infrared

FTIR analysis of the synthesized nanomaterials was done at Bahir Dar University Chemistry Department. FTIR spectrum of the dried powder of the biosynthesized α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite, and *Hagenia abyssinica* leave extract samples were recorded on JASCOFT/IR-6600 type A in the range 4000-400 cm⁻¹.

FT-IR is used to identify the functional groups of the active components responsible for synthesizing α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite and ZnO/ α -Fe₂O₃/Bentonite NCs (Zewudie et al., 2023).

Sample preparation: The synthesized NPs and NCs α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite and Hagenia abyssinica leaf extract samples powders of the nanomaterials were mixed with potassium bromide (KBr) and diluted to form a very fine powder. The powder was then compressed into a thin transparent pellet which was used for recording the FTIR spectrum. The procedure is the same for leaf powder.

3.5.4. UV-Vis Spectroscopy

The optical properties and band gap energy of synthesized magnetite nanoparticles were characterized by Uv-Vis DRS Elico SL-150 spectrophotometer at Adama Science and Technology University Material Science Department. The range of absorption was between 200 nm to 800 nm (Garshasbi et al., 2017; Saied et al., 2022; Vaizoğullar, 2018b).

Sample Preparation: A sample container with a rectangular or circular opening and a surface area of a few tens square millimeters to several square centimeters was carefully filled with finely ground standard (BaSO₄) and sample powder. The diffuse reflectance spectra were then taken after the holder was positioned horizontally at the base of the integrating sphere.

3.5.5. Thermo gravimetric analysis

Thermo gravimetric analysis of the synthesized NPs and NCs of α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite were done at Adama Science and Technology University Material Science Department in a flowing air atmosphere and the thermograms were recorded in the temperature range of 25–800 °C, at a heating rate of 20 °C/min. TGA measures the percent weight loss of a test sample (Moharram et al., 2014).

Sample Preparation: A piece of powder specimen (10 mg) was placed in a platinum basket, and a thermocouple beneath the basket continually monitored the temperature. TGA calculates the percent weight loss of a test sample while it was heated evenly in the proper atmosphere.

3.5.6. Photoluminescence analysis

Separation efficiency electron and hole pair of the synthesized NPs and NCs of α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite determined in PL spectrophotometer of a JASCD FB-8500 done at Adama Science and Technology University Applied Physics Department of the shows an intense emission when excited with λ_{max} radiation

Sample Preparation: Finely ground powder of α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite dissolve by DMSO then packed into a Uv-Vis sample holder then the holder was placed in PL spectrophotometer and excited wavelength was recorded.

3.5.7. Cation exchange capacity analysis

Cation exchange capacity (CEC) is usually estimated by displacing the exchangeable cations with another strongly adsorbed cation, and then determining how much of the strongly adsorbed cation is retained by the bentonite done at Adama Science and Technology University Applied chemistry laboratory.

Sample Preparation: The methylene blue adsorption method was a simple and quick way to measure the cation exchange capacity value of bentonite. The method involves diluting the methylene blue solution with water and stirring it with a magnetic stirrer for one minute. Then, 3 mL of the methylene blue solution was added to the sample in a glass beaker and stirred for one minute. After one minute, titration with methylene blue solution is performed, and any change during the titration was followed by dripping on filter paper to see a light blue halo around dark blue. This indicates the substitution between bentonite and methylene blue, and the absorption continues, which is the principle of the methylene blue method. The analysis is repeated three times to ensure accuracy. The CEC values can be estimated using this method and calculated by equation (2).

$$CEC(\text{meq per 100g of clay}) = \frac{100}{F \times V \times NMB} \quad \text{Equation 2}$$

Where F is the sample weight, V is the required methylene blue volume at the time of titration, Normality of methylene blue (NMB) 0.028 N.

3.6. Analysis of antibacterial activity

The disk diffusion method is one of the most popular approaches to bacterial sensitivity testing due to its low cost, efficiency, commercially available and contains the proper concentrations of antibiotic drugs recommended for susceptibility testing by the Clinical and Laboratory Standards Institute (CLSI), the institution responsible for maintaining standards for such research. The antibacterial activity of the ZnO, α -Fe₂O₃, ZnO/bentonite, α -Fe₂O₃/bentonite, and ZnO/ α -Fe₂O₃ /bentonite against both *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) will be tested by agar diffusion test. Samples will be exposed to bacteria in solid media (nutrient agar), and the inhibition zone around each sample will be measured and recorded as the antibacterial effect of prepared NCs. Agar plates will be inoculated with 100 μ L suspensions of bacteria. NCs were placed on 0.5 cm diameter agar disks and incubated at 37 °C for 24 hours. The inhibition zone for bacterial growth were measured (Garshasbi et al., 2017).

3.7. Analysis of antifungal activity test

To evaluate the antifungal activity of synthesized α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite NPS and NCs against *Candida* strains, including *Albicans* (ATCC 10231), a standard disk diffusion method was used. The candida suspension was prepared using 0.5% DMSO solution, and the microbial turbidity was adjusted using Mueller–Hinton agar (MHA) medium supplemented into sterile Petri dishes. The prepared microbial suspension was streaked over the poured MHA plates using sterile swabs. The dried α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite NPS and NCs were suspended in DMSO solvent, and sterile filter paper disks (8 mm in diameter) were impregnated with the dissolved α -Fe₂O₃, ZnO, and bentonite, ZnO/bentonite, α -Fe₂O₃/bentonite, ZnO/ α -Fe₂O₃/Bentonite nanomaterials to attain concentrations of 10, 5, and 2.5 mg/mL (Strains et al., 2022). The antifungal effect was determined by measuring the clear zones that corresponded to the inhibition formed around the nanoparticles. Ketoconazole was used as a standard antifungal agent, and filter paper disks impregnated with DMSO solvent only were used as negative controls.

3.7.1. Determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC)

The bactericidal and fungicidal effect of attractive α -Fe₂O₃/ZnO/Bentonite NCs was assessed using a microbial culture selected for the study. The minimum inhibitory concentration (MIC), which is the lowest concentration of material that inhibits the growth of an organism, was determined using the serial dilution method. 10 test tubes were filled with 2 mL of liquid Broth Agar medium, and the nanocomposite solution was added serially to each tube, starting with a concentration of 20 mg/mL in tube 1 and decreasing by a factor of 2 in each subsequent tube. Microbial suspensions of *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* were then added to each tube, and the tubes were incubated at 35 °C for 24 hours. The growth of the microorganisms was observed by turbidity measurement. Positive and negative controls were included in the experiments to indicate the microbial growth profile in the presence and absence of nanocomposites.

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), which is the lowest concentration of nanoparticles that kills 99.9% of the bacteria and fungi, were also determined. The presence of viable microorganisms was tested for the growth inhibitory concentration (MIC), and the lowest concentration causing bactericidal and fungicidal effects was reported as MBC and MFC. To assess the bactericidal and fungicidal effect, in column from each test tube were plated on Muller Hinton Agar and incubated at 35 °C for 24 hours. The concentration of nanoparticles that resulted in the absence of colonies on the agar plate was considered to illustrate the bactericidal effect. All experiments were performed in triplicate reported as MBC and MFC (Chikezie, 2017). For experiments for bactericidal and fungicidal effects, each test tube (Especially, negative and positive test tubes) was inoculated on Muller Hinton Agar and incubated at 35 °C for 24 hr. The nanoparticles concentration illustrating the bactericidal effect was picked out based on the absence of colonies on the agar plate (Zewudie et al., 2023).

3.8. Statistical Data Analysis

Data in the current study (Antibacterial activity data) were analyzed by Microsoft Excel 2010 software. The means of three replications and standard error ($\pm$ SE) were calculated for all the results obtained. Differences between means were considered significant at P-values < 0.05 .

3.9. Software for data analysis

Origin Pro 8, Image j, Microsoft Paint, QJIS, and Excel software were used to analyze the data.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Phytochemicals test of *H. abyssinica* plant leaf extract

Screening for phytochemicals is an important process that helps to identify the different types of compounds present in the selected plant leaf aqueous extract. In this study, the researchers used the naturally occurring phytochemicals found in *H. abyssinica* plant leaf extract to reduce and capping for the synthesis of targeted materials. The phytochemicals test of *H. abyssinica* was conducted using standard protocols and the results revealed the presence of secondary metabolites such as phenols, flavonoids, carbohydrates, alkaloids, and others as listed in Table 1. These phytochemicals are capable of reducing and capping iron oxide and zinc oxide NPs by donating electrons and stabilizing the formed NPs.

Observed color changes during qualitative phytochemical tests for active compounds in the plant leaves extract of *H. abyssinica* is shown by Figure 10. The color changes against the reagents indicates the formation of different complexes due to oxidation and reduction reactions. For instance, the yellow color of the alkaloid indicates that the nitrogen or oxygen atoms of the amide groups of the alkaloids are involved in a reaction. The different colors observed in the ferric chloride tests indicate the formation of complexes with different properties depending on the nature of the complexes (Zewudie et al., 2023).

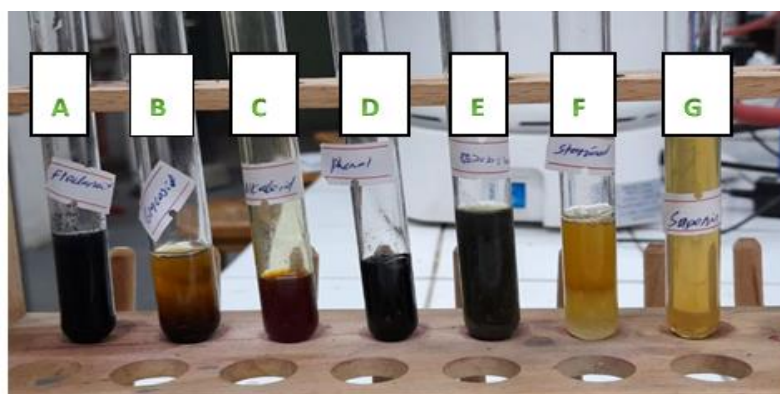


Figure 10. Results of qualitative tests for phytochemical screening.

Where: a, b, c, d, e, f, and g are pictures of the tests undertaken for Flavonoids, alkaloids, glycosides, steroids, phenol, carbohydrate, and saponins respectively.

Table 1.Phytochemical analysis of *H. abyssinica* leaves aqueous extract.

S. No	Phytochemical	Name of the Test	Result
1	Saponins	Foam test	+
2	Alkaloids	Wagner	+
3	Phenols	FeCl ₃ test	+
4	Steroids	Chloroform test	+
5	Flavonoids	NaOH test	+
6	Glycosides	Salkowski's test	-
7	Carbohydrate	Fehling's test	+

Note: + = Present and – = Absent

4.2 Cation exchange capacity analysis of Bentonite

The methylene blue method is a widely used technique to determine the cation exchange capacity (CEC) value of bentonite. The CEC value of clay minerals is a measure of their ability to adsorb cations from solution, and a higher CEC value indicates better performance in various applications (Rihayat et al., 2018). In this study, the CEC values of raw and activated bentonite samples were determined and compared. The results showed that the CEC values of raw and activated bentonite were significantly different, from the activated bentonite having an improved CEC value ranging from 51.02 to 102.04 meq g⁻¹. The results of this study indicated that the sodium carbonate-activated bentonite had a higher CEC value than natural ground bentonite. This suggests that the activation process enhances the cation exchange capacity of bentonite. The CEC value of bentonite can be influenced by several factors such as the presence of exchangeable cations, the concentration of Na₂CO₃, and the content of montmorillonite. It is noted that sodium-rich bentonite tends to have higher CEC values, better swelling properties, and improved performance in various applications. These findings also highlight the importance of activation in improving the CEC value of bentonite. and suggest potential applications for this material in the antifungal and antibacterial application (M. Kumar et al., 2021).

4.3 Analysis of XRD

4.3.1 XRD patterns of Bentonite sample

The changes in the structure and layer spacing of raw bentonite before and after acid activation were studied using X-ray diffraction. Figure 11 shows the X-ray diffraction results for activated bentonite, raw bentonite, and purified bentonite clay. The diffraction peaks for activated bentonite were observed at around 8.65°, 15.84°, 19.74°, 26.06°, 27.58°, 29.42°, 30.66°, 39.48°, 56.62°, 61.54°, and 72.94°. The diffraction peaks for purified bentonite were observed at around 8.62°, 15.78°, 19.79°, 25.76°, 27.88°, 29.58°, 30.32°, 39.42°, 56.48°, 61.59°, and 72.94°. The diffraction peaks for raw bentonite clay were observed at around 8.50°, 15.86°, 19.76°, 26.06°, 27.88°, 29.44°, 30.62°, 39.44°, 56.64°, 61.56°, and 72.94°. These diffraction peaks correspond to the crystal planes of (001), (211), (400), (101), (332), (431), (521), (640), and (921). According to the crystal data sheet JCPDS00-041-1478, these results are consistent with those reported by (Ahmad & Yasin, 2020). The X-ray diffraction pattern also shows the presence of low quartz and silicon oxides at some absorption, which is also consistent with the XRD analysis.

As shown in Figure 11, RB, PB, and AB samples contain montmorillonite and quartz, confirming the presence of the smectite phase. The XRD pattern of the RB sample exhibited a well-defined and intense "001" peak at a 2θ value of 8.77° (d (001) spacing of 10.06 Å). Similarly, the XRD pattern of the purified bentonite sample showed a similar "001" peak at a 2θ value of 8.64° (d (001) spacing of 10.22 Å), but the peak was slightly broader and less intense, indicating that the purification process had caused some damage to the montmorillonite crystal structure. The XRD pattern of the activated bentonite sample showed a very broad "001" peak at a 2θ value of 8.62° (d (001) spacing of 10.25 Å), suggesting that the activation process had caused significant damage to the montmorillonite crystal structure. Additionally, the peak was shifted to a lower 2θ value, indicating that the d (001) spacing had increased. This is consistent with the fact that activation is known to cause an expansion of the interlayer space in montmorillonite clays, as reported by (Maged et al., 2020).

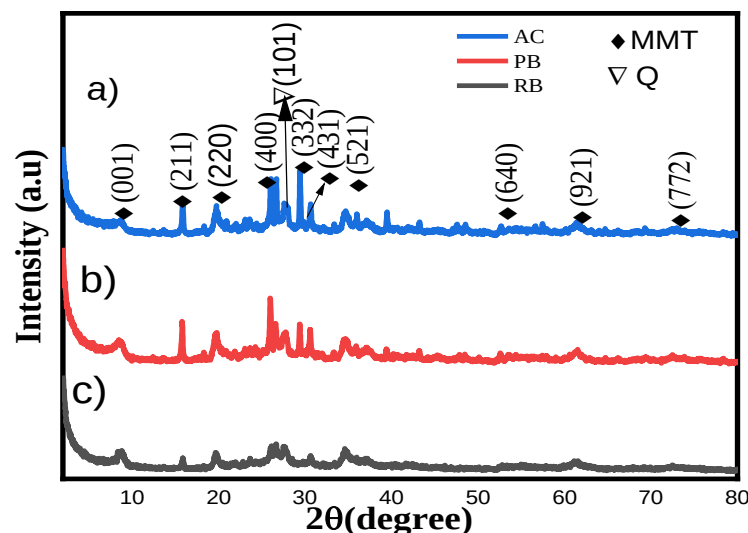


Figure 11. XRD Patterns of a) AB (Activated Bentonite), b) PB (Purified Bentonite), and c) (RB) Raw Bentonite, where MMT: Montmorillonite and Q: quartz

Table 2. Results of interlayer spacing and composition of PB, Na-AB, and RB

Sample	Miller indices(hkl)	Strong Peak position (2θ(°))	d-space (Å)	Indication
Activated Bentonite	(211)	15.84	3.42	MMT
	(220)	19.74	4.48	MMT
	(400)	26.06	3.34	MMT
	(101)	27.58	3.03	Q
Purified Bentonite	(211)	15.78	3.41	MMT
	(220)	19.79	5.56	MMT
	(400)	25.76	2.91	MMT
	(101)	27.88	3.03	Q
Ground Bentonite	(211)	15.86	3.41	MMT
	(220)	19.76	3.02	MMT
	(400)	26.06	3.21	MMT
	(101)	27.88	3.02	Q

4.3.2 XRD patterns of ZnO Nanoparticles

The XRD patterns of ZnO NPs with different ratios of 2:1, 1:2, and 1:1 can be seen in Figure 12. The patterns showed similar peak positions and intensity distributions, confirming the hexagonal wurtzite structure of the synthesized nanoparticles. For the 1:2 ratios, diffraction peaks were observed at 2θ values of 31.74°, 34.44°, 36.32°, 47.68°, 56.6°, 62.98°, 66.54°, 68.12°, and 69.52°. For the 2:1 ratio, peaks were found at 2θ values of 31.82°, 34.46°, 36.26°, 47.66°, and 69.52°.

56.62°, 63.02°, 66.36°, 68°, and 69.1°. Finally, for the 1:1 ratio, peaks were observed at 2θ values of 31.72°, 34.44°, 36.26°, 47.6°, 56.68°, 62.92°, 66.34°, 68.12°, and 69.22°. These results are consistent with previous research (Suresh et al., 2018) and suggest that the ratio of precursors used in the synthesis of ZnO NPs significantly affects their crystal structure corresponding to a crystal plane of (100), (002), (101), (102), (110), (103), (200), (112), and (201) as shown in Figure 12.

The XRD pattern of ZnO NPs reveals a highly crystalline and pure hexagonal wurtzite structure, with no detectable impurities. This data is consistent with previous research and confirms the quality of the nanoparticles. The FWHM results of $d(100) = 2.812 \text{ \AA}$, $d(002) = 2.7676 \text{ \AA}$, and $d(101) = 3.6011 \text{ \AA}$ further support the high crystallinity and purity of the NPs and space group of P63mc. This data is also in good agreement with the Joint Committee on Powder Diffraction Standards (JCPDS card no 00-036-1451) (Dobrucka & Długaszewska, 2016).. The average size of the crystallites in ZnO NPs with ratios of (1:2), (2:1), and (1:1) was estimated to be 22.7, 27.3, and 26.1 nm, respectively. Interestingly, the use of plant extract was found to be responsible for the reduction or capping of the NPs, and the size of the NPs decreased with an increasing amount of the plant extract (Zewudie et al., 2023).

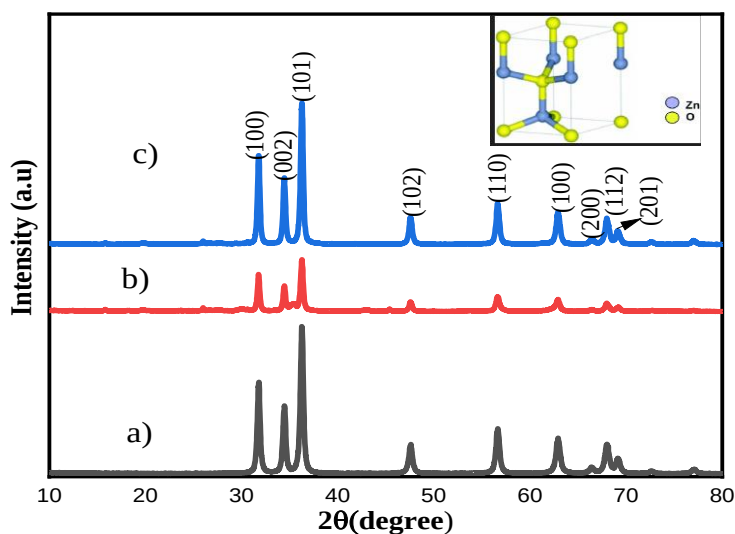


Figure 12. XRD diffraction patterns of synthesized a) (2:1), b) (1:2), and c) (1:1) ZnO NPs.

4.3.3 XRD patterns of ZnO/Bentonite Nanoparticles

The XRD diffraction spectrum of biosynthesized ZnO/Bent NCs in different ratios (1:1, 1:2, and 2:1) is illustrated in Figure 13. The spectrum displays characteristic peaks of both ZnO and bentonite phases in the NCs. The ZnO peaks correspond to Miller indices of (100), (002), (101), (102), (110), (103), (112), and (004), while the bentonite peaks correspond to Miller indices of (220), (400), (332), (640), (921), and (772), respectively and Bentonite characteristic peaks are shown at 2θ values of 15.94, 19.86, 26.1, 30.7, 52.72, 68.06, and 77.12°, while the prominent ZnO peaks are observed at 2θ of 31.8°, 34.58°, 36.32°, 47.66°, 56.72°, 62.92°, 69.32°, and 72.54° (Vaizoğullar, 2018a). The diffraction peaks observed in the XRD pattern of the composite material can be attributed to the face-centered cubic (FCC) structure of ZnO NPs. This identification is supported by the JCPDS card no 00-036-1451, which confirms the presence of ZnO nanoparticles in the composite and activated bentonite shows reflections that match the JCPDS card no. 00-041-1478. The XRD pattern of ZnO/bentonite NPs clearly reveals that the composite peak intensity and interlayer spacing value increase when a high concentration of ZnO solution (2:1) is present. Conversely, the composite peak intensity and interlayer spacing value decrease in ZnO /bentonite nanocomposites with a low concentration of ZnO solution (1:2) and equal ratio in (1:1).

However, reducing the amount of ZnO content leads to the formation of smaller NPs. This could be attributed to the sufficient amount of bentonite clay that acts as a capping agent and prevents ZnO NPs from aggregating which indicates (1:1). Additionally, the decreased intensity of ZnO XRD peaks indicates the NCs ZnO/Bent at 1:1 and 1:2 are less crystalline which means low intensity and XRD peak broadening can be attributed to homogeneities of micro-strain in the crystal lattice of ZnO become distorted due to dislocation and increased in a concentration gradient of bent became increased, hence, as bentonite increased from 1 to 2 ratio lattice distortion become more intense, manifested as broadening and loss of peaks (de Lucas-Gil et al., 2020). In shown in Appendix C, it is shown that the crystallite size of (2:1) and (1:1) ZnO decreases from 27.3 to 12.2 nm with an increase in the concentration of bentonite from 1 to 2. This implies that as the diffraction peak becomes narrower and more distinct, the crystallite size increases (Yang et al., 2021).

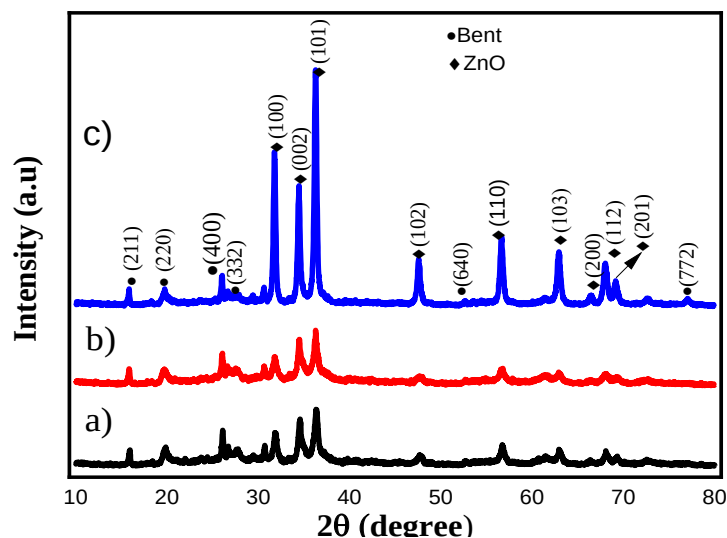


Figure 13. XRD diffraction patterns of synthesized a) (1:1), b) (1:2), and c) (2:1) ZnO/Bent.

4.3.4 XRD patterns of α -Fe₂O₃ Nanoparticles

The XRD analysis was used to determine the phase purity and crystallinity of magnetite nanoparticles synthesized using two different ratios of metals to plant extract: (1:2) and (1:1). The XRD patterns of both samples showed similarity in peak position and intensity distribution can be seen in the Figures 14. For the (1:2) ratio of α -Fe₂O₃ NPs, diffraction peaks were observed at 2θ values of 24.22°, 33.12°, 35.66°, 40.88°, 43.44°, 49.48°, 54.04°, 57.58°, 62.41°, 64.12°, 72.5°, 75.66°, and 77.66°, corresponding to the crystal plane of (012), (104), (110), (133), (202), (024), (116), (122), (214), (300), (208), (1010), (220), and (306), respectively. Similarly, for α -Fe₂O₃ (1:1) NPs, diffraction peaks were observed at 2θ values of 24.26°, 33.18°, 35.71°, 40.94°, 43.94°, 49.54°, 54.1°, 57.58°, 62.52°, 64.26°, 64.12°, 69.54°, 71.94°, 75.5°, and 77.54°, corresponding to the crystal plane of (012), (104), (110), (133), (202), (024), (116), (122), (214), (300), (018), (208), (1010), and (220), respectively. These results demonstrate the high crystallinity and phase purity of the synthesized magnetite nanoparticles, which are important properties for various applications (Lassoued et al., 2020).

The diffraction pattern of α -Fe₂O₃ NPs was found to be in good agreement with JCPDS card number 00-033-0664 which confirms the formation of α -Fe₂O₃ NPs. The XRD analysis was performed on both ratios of α -Fe₂O₃ NPs to determine their crystal structure. The XRD patterns showed peaks that matched the expected rhombohedral (hexagonal) structure of α -Fe₂O₃ (space group: R-3c), with lattice constants of $a = 0.5034$ nm and $c = 1.375$ nm. The prominent peak observed at $2\theta = 33.18^\circ$ in the as-prepared sample corresponded to the reflection from the (104) plane of the α -Fe₂O₃ lattice, confirming the presence of this phase.

However, the presence of reducing agents in the extracted plant material used in the synthesis process may have resulted in the formation of additional peaks in the XRD pattern of the sample, specifically at 31.76° . These peaks may correspond to the (220) plane of magnetite (Fe₃O₄), which is a common impurity that can coexist with hematite in some iron oxide samples. The peak at 37.62° may correspond to the (311) plane of wüstite (FeO), which is another possible impurity that can be present in iron oxide samples (Justus et al., 2021). Figure 14 shows that hematite has a rhombohedral crystal structure with a hexagonal unit cell and the calcination process can increase its peak intensity and sharpness, indicating higher crystallinity. Increasing the precursor concentration led to a gradual decrease in the intensity of the (104) diffraction peak and an increase in particle size. The size of the green synthesized magnetite NPs was found to decrease with an increase in the amount of plant extract used, indicating its role in reducing or capping the NPs. The crystal size of the highest intense peaks (1:1) and (1:2) was found to be 28.7 nm and 20.2 nm, respectively (Nurdini et al., 2022).

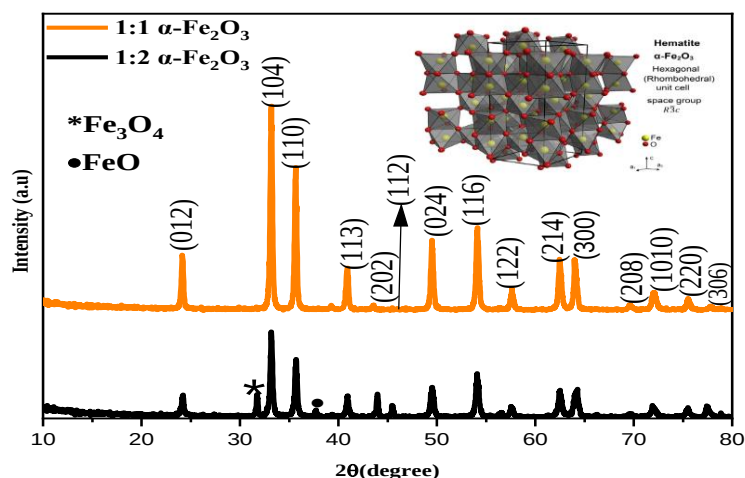


Figure 14. XRD diffraction patterns of synthesized α -Fe₂O₃ NPs.

4.3.4 XRD patterns of α -Fe₂O₃/Bentonite Nanocomposite

The XRD diffraction spectrum of biosynthesized α -Fe₂O₃/Bentonite NCs in different ratios (1:1, 1:2, and 2:1) reveals the presence of crystalline phases of both α -Fe₂O₃ and Bentonite. The FCC (spinel structure) phase of α -Fe₂O₃ has a lattice parameter of 8.35 Å, and its XRD peaks are located at specific 2θ values (24.26°, 33.18°, 35.72°, 40.96°, 49.48°, 54.06°, 57.52°, 62.46°, 63.08°, 66.18°, 71.94°, 75.58°, and 77.94°). In addition, the mono clinic phase of Bentonite has specific lattice parameters ($a = 5.17$ Å, $b = 8.94$ Å, $c = 10.15$ Å, and $\beta = 97.7^\circ$), and its XRD peaks are located at 2θ values (15.88°, 19.74°, 26.08°, 30.66°, 34.68°, 37.16°, 52.7°, and 69.68°), as illustrated in Figure 15 (Wang, 2018). These with JCPDS card number 00-033-0664 confirm the formation of α -Fe₂O₃ NPs which confirms the presence of α -Fe₂O₃ NPs in the composite and activated bentonite also shows reflections that match the JCPDS card number.00-041-1478. The XRD pattern of the biosynthesized α -Fe₂O₃/Bentonite NCs confirms the successful formation of the composite in a three-volume ratio. The pattern displays a sharp and intense peak, indicating that the sample has been finely ground, homogenized, and possesses high crystalline quality. Furthermore, the average crystallite sizes and lattice strain values were determined using Debye Scherrer's formula, revealing that the calculated average crystallite sizes for the α -Fe₂O₃/Bentonite NCs in powder form were 20.2, 20.4, and 30.1 nm for volume ratios of 1:1, 1:2, and 2:1, respectively.

The XRD pattern of α -Fe₂O₃-bentonite clearly shows that in the presence of a high concentration of iron oxide solution (2:1), the composite characteristics peak intensity and interlayer spacing value increase, whereas the composite characteristics peak intensity and interlayer spacing value decrease in α -Fe₂O₃-bentonite NCs with a low concentration of iron oxide solution (1:2). However, decreasing amount of α -Fe₂O₃ content is accompanied with formation of smaller size NCs. This might be due to more enough amount of bentonite clay responsible for supportive and preventing α -Fe₂O₃ NPs from agglomeration (Křišťan et al., 2013). The average crystallite size of the α -Fe₂O₃/Bentonite NCs in the 1:1 volume ratio is smaller than that of the 1:2 and 2:1 ratio, with a size of 20.2 nm.

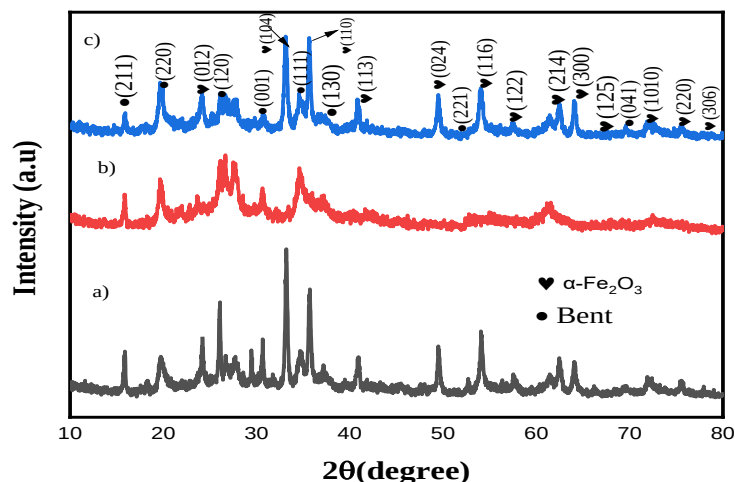


Figure 15. XRD diffraction patterns of synthesized a) (2:1), b) (1:2), and c) (1:1) α - Fe_2O_3 /Bentonite

4.3.4 XRD patterns of ZnO/ α - Fe_2O_3 /Bentonite Nanocomposite

After intercalating nanoparticles of α - Fe_2O_3 and ZnO in different ratios of clay, the presence of additional peaks corresponding to the formation of α - Fe_2O_3 and ZnO in the composites was observed. Specifically, the peaks were reflected at $2\theta = 26.1^\circ$ for α - Fe_2O_3 , corresponding to the (104) crystallographic planes of the face-centered cubic (FCC) α - Fe_2O_3 crystals (Farahmandjou & Soflaee, 2015). Additionally, peaks were observed at $2\theta = 32, 34$, and 36° , corresponding to (100), (002), and (101) planes, respectively, showing the presence of the wurtzite phase of ZnO (Garshasbi et al., 2017). The XRD spectrum of pure α - Fe_2O_3 NPs shows reflections that match the Rhombohedral structure, which is consistent with the JCPDS Card No. 00-033-0664 (Saied et al., 2022). Similarly, the observed XRD peaks for ZnO NPs are consistent with the hexagonal wurtzite structure with the P63mc space group, in agreement with the JCPDS Card No. 01-036-1451 (Sridharan, 2023). The activated bentonite also shows reflections that match the JCPDS card no. 00-041-1478 (A. Kumar & Lingfa, 2020). The diffraction patterns of α - Fe_2O_3 , ZnO, and bentonite confirmed that the prepared composites are composed of these three species. The ZnO/ α - Fe_2O_3 /Bentonite NCs were synthesized in three different volume ratios, and their crystal structure and properties were studied.

The 1:1:1 ratio had the smallest average crystallite size of 10.36 nm compared to the other two ratios (2:2:1 and 1:1:3), which indicated that equal amount α -Fe₂O₃, ZnO and bentonite as solid support matrix responsible for capping and preventing α -Fe₂O₃ and ZnO from agglomeration. Due to the intercalation of NPs into Na-Bentonite caused structural changes due to electrostatic attraction and hydrogen bonding forces between Na-Bentonite and intercalated NPs. This led to a decrease in D values in the nanocomposites. The diffraction patterns of the ZnO/ α -Fe₂O₃/Bent NCs formed in a 2:2:1 volume ratio had sharp peaks and high intensity, indicating a large average crystalline size compared to the other two ratios as shown in Figure 16. This was because this ratio had a higher amount of the precursor of metal oxide in solid support matrix (Abbas et al., 2020b). Also, in the (1:1:2) ratio ZnO and α -Fe₂O₃ with weak intensity due to the small amount of both NPs as shown in Figure 16.

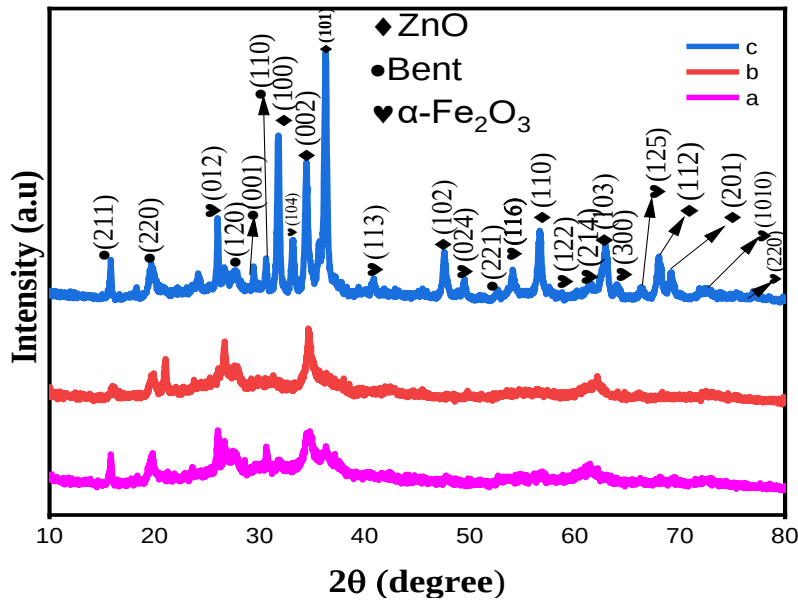


Figure 16. XRD diffraction patterns of synthesized a) (1:1:1), b) (1:1:2) and c) (2:2:1) ZnO/ α -Fe₂O₃/Bentonite.

Table 3. Results of interlayer spacing and composition

Samples	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:1:1ZnO/ α -Fe ₂ O ₃ /Bent	34.7	2.6	1.032	0.999	7.701	10.36	(100)
	26.1	3.4	0.646	0.999	12.290		(104)
	19.8	4.8	0.715	0.999	11.116		(114)
1:1:3ZnO/ α -Fe ₂ O ₃ /Bent	34.7	2.6	0.608	0.999	13.060	16.04	(111)
	26.7	3.3	0.537	0.999	14.798		(101)
	21.1	4.2	0.391	0.999	20.281		(221)
2:2:1ZnO/ α -Fe ₂ O ₃ /Bent	36.3	2.5	0.301	0.999	26.362	28.87	(100)
	26.1	3.4	0.226	0.999	35.060		(110)
	34.5	2.6	0.315	0.999	25.200		(101)

4.4 Scanning electron Microscope Analysis

Scanning electron Microscope Analysis (SEM) images of bentonite, ZnO NPs, α -Fe₂O₃ NPs, α -Fe₂O₃/Bentonite, ZnO/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite which were taken at different magnification are displayed by Figure 17. The images revealed that the ZnO NPs and α -Fe₂O₃ NPs have spherical shapes to some extent agglomerated, which might be due to the interactions and van der Waals forces between the particles. These findings provide important insights into the morphology and structure of the synthesized materials. The SEM images of α -Fe₂O₃/Bentonite, ZnO/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs formed on bentonite by using *H. abyssinica* leaf extract were more or less similar to the SEM images of bentonite. The biomolecules of the leaf extract used for the synthesis of different NPs (ZnO and Fe₂O₃) resulted morphologies as observed in Figure 17 B.

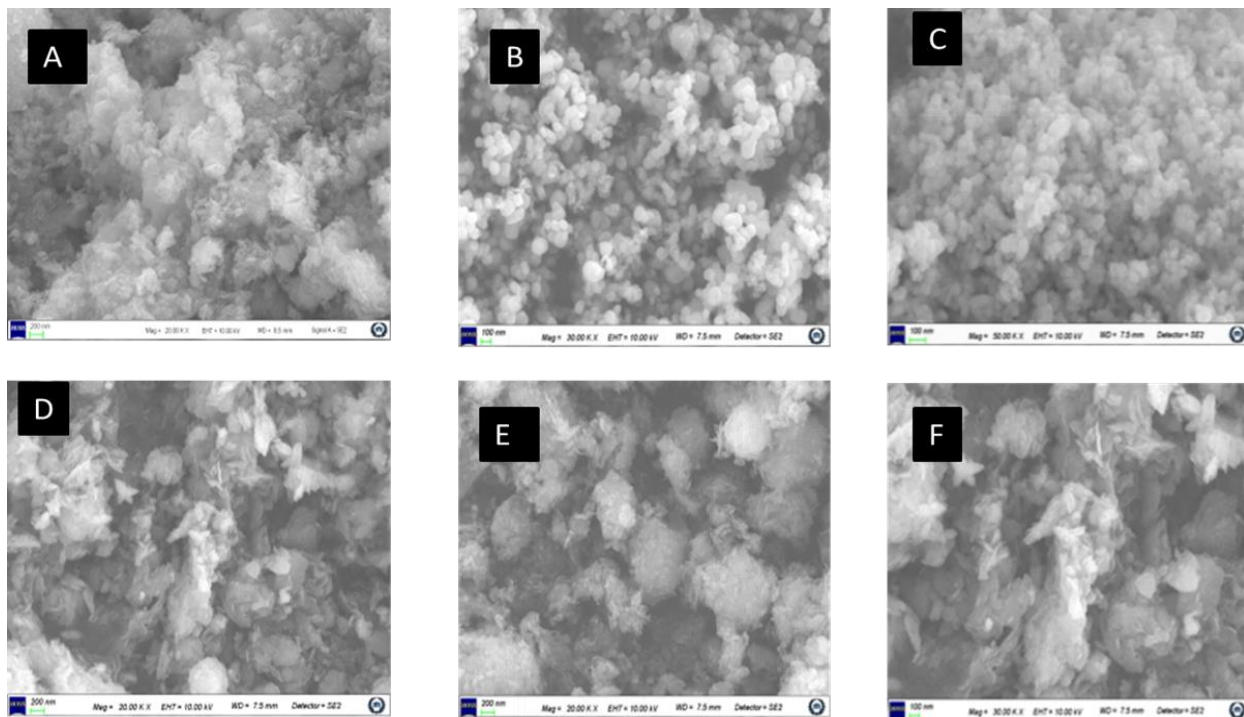


Figure 13. SEM image of the synthesized (A) Activated bentonite, (B) (1:2) ZnO NPs, (C) (1:2) α -Fe₂O₃ NPs, (D) (1:1) ZnO/Bentonite, (E) (1:1) α -Fe₂O₃/Bentonite, and (F) (1:1:1) ZnO/ α -Fe₂O₃/Bentonite NPs respectively.

4.5 UV-visible diffuse reflectance spectra and band gap analysis

The optical properties of the produced material were analyzed using UV-Vis diffuse reflectance spectroscopy (DRS), and the results are presented in Figure 18. The band gap (E_g) of the samples was determined using the Kubelka-Munk function.

$$(F(R)h\nu)^n = [B(h\nu - E_g)] \quad \text{Equation 3}$$

To determine the band gap energy of the synthesized NPs and NCs, the energy of the photon ($h\nu$) was calculated, where h represents Planck's constant ($h = 6.63 \times 10^{-34}$ J.s), B is a constant, E_g is an optical gap energy, $F(R)$ is the optical absorption coefficient and ν is the frequency of the photon. For direct allowed transition, $n = 2$, while for indirect allowed transition, $n = 1/2$ (Kerchich et al., 2020).

The change in $(F(R) h\nu)^2$ vs $h\nu$ for the sample indicated that the energy gap of the direct band gap could be obtained by extrapolating the linear part to the $h\nu$ axis. The band gap energy of the MO NPs synthesized using a green method with a plant leaf extract was analyzed using UV-Vis DRS, as shown in Figure 18. For instance, the presence of magnetite nanoparticles was confirmed by the immediate change in color of the reaction mixture from light yellow to dark brown after the addition of *H. abyssinica* plant leaf extracts (Shafiee et al., 2020). The band gap energy results obtained from the α -Fe₂O₃NPs with volume ratios of 1:1 and 1:2 was 2 and 2.03 respectively, as shown in Figure 18. It was observed that the 1:2 volume ratios of α -Fe₂O₃ nanoparticles had a larger band gap energy compared to the 1:1 ratio. This suggests that the energy band gap of α -Fe₂O₃ nanoparticles is related to their particle size, with smaller particles having larger band gaps (Kerchich et al., 2020).

In Figure 18 (1:1 ZnO) and (1:2 ZnO), the biosynthesized ZnO NPs with different ratios of plant and precursor exhibited varying energy band gaps of 3.21 and 3.25, respectively. This variation in the energy band gap suggests that different types of the synthesized ZnO nanoparticles absorb at different regions of UV-Vis light (Jayachandran et al., 2021). The analysis of band gap energy confirmed the successful formation of ZnO NPs, which is consistent with previously reported values (Han et al., 2019). From the band gap energy graph, it can be observed that as the band gap energy decreases, the average crystalline size of the NPs increases, leading to enhanced properties. The band gap energy of various NCs, including (1:1) ZnO/Bentonite, (1:2) ZnO/Bentonite, (1:1) α -Fe₂O₃/Bentonite, (1:2) α -Fe₂O₃/Bentonite, (1:1:1) ZnO/ α -Fe₂O₃/Bentonite, and (1:1:2) ZnO/ α -Fe₂O₃/Bentonite, were found to be 2.97, 3.19, 2.03, 2.04, 3.23, and 2.98, respectively, as shown in Figure 18. These results suggest that the band gap of the NCs is dependent on the crystal size and that zinc oxide composite without α -Fe₂O₃ and bentonite exhibits almost no light absorption in the visible light region. However, bentonite can absorb visible light, leading to greater absorption of visible light in ZnO/Bentonite and α -Fe₂O₃/Bentonite compared to pure ZnO and α -Fe₂O₃. Additionally, as the concentration of the precursor increases, the band gap of the NCs decreases (Klink et al., 2022). The results showed that (1:1:2) ZnO/ α -Fe₂O₃ has the smallest band gap, indicating that its electrons can be easily excited from the valence band to the conduction band under visible light irradiation.

Therefore, α -Fe₂O₃ reduces the band gap of ZnO, extending its light absorption from the ultraviolet to the visible light region. Meanwhile, bentonite can absorb visible light as well. (1:1:1) ZnO/ α -Fe₂O₃/Bent has a higher band gap and smaller crystal size, making it more suitable for antimicrobial applications. Relatively as indicated in Figure 18 the reflectance values of different NPs and NCs at different wavelengths were measured. For instance, when pure α -Fe₂O₃ and ZnO NPs were analyzed, the reflectance values at wavelength were 610 nm, 607 nm, 388.9 nm and 386.3 nm respectively for ratios of 1:1 and 1:2 of α -Fe₂O₃ and ZnO NPs. When the NCs of ZnO/bentonite were analyzed, the reflectance values were 283.9 nm and 417 nm for ratios of 1:1 and 1:2 of ZnO to bentonite, respectively. Similarly, when the NCs of α -Fe₂O₃/bentonite were analyzed, the reflectance values were 607.8 nm and 600 nm for ratios of 1:1 and 1:2 of α -Fe₂O₃ to bentonite, respectively. Finally, ZnO/ α -Fe₂O₃/bentonite was analyzed; the reflectance values were 381.53 and 416.10 for ratios of 1:1 and 1:2 of ZnO to α -Fe₂O₃, respectively. These values suggest that increasing the amount of α -Fe₂O₃ in the mixture slightly increases the reflectance of the nanoparticles.

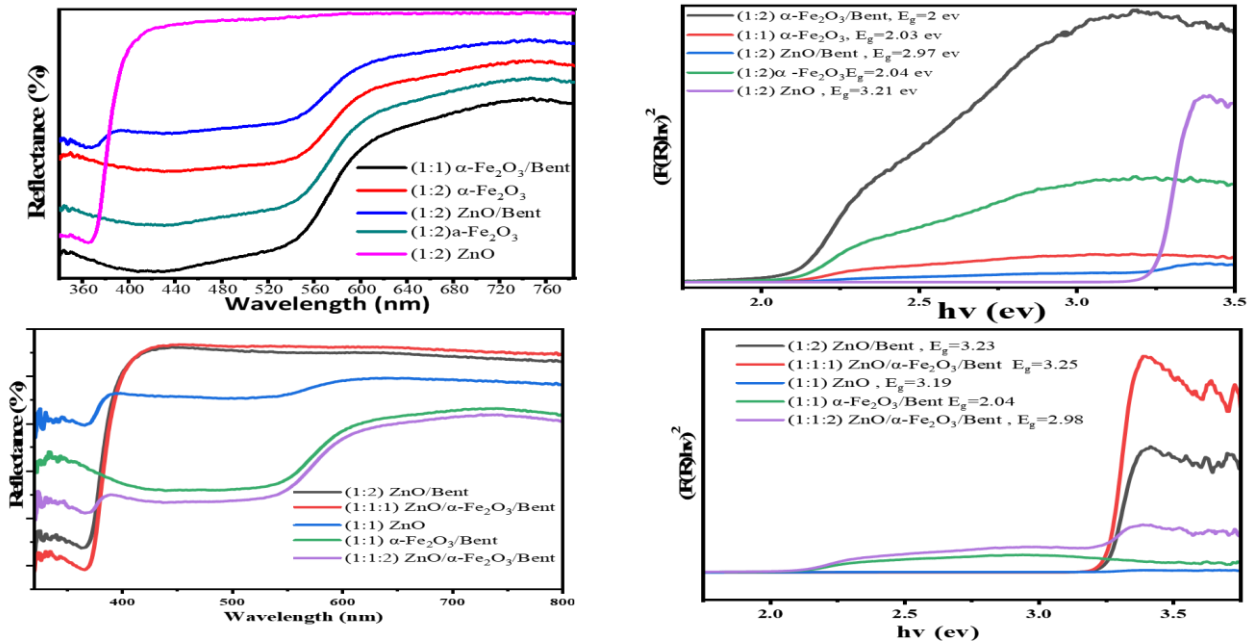


Figure 14. DRS spectra of (1:1) ZnO, (1:2) ZnO, (1:1) ZnO/Bentonite, (1:1) α -Fe₂O₃, (1:2) α -Fe₂O₃, (1:2) ZnO/Bentonite, (1:1) α -Fe₂O₃/Bentonite, (1:2) α -Fe₂O₃/Bentonite, (1:1:1) ZnO/ α -Fe₂O₃/Bentonite and (1:1:2) ZnO/ α -Fe₂O₃/Bentonite and its corresponding band gap energy.

4.6 Thermo Gravimetric Analysis

Thermal gravimetric analysis (TGA) is a powerful tool for studying the thermal stability and decomposition behavior of materials. The TGA curve of the synthesized α -Fe₂O₃ by the precipitation method showed three distinct mass loss steps occurring over different temperature ranges (Fouad et al., 2019). The first weight loss step occurred gradually between [31.4–119 °C], with a mass loss of 0.34%. This weight loss is attributed to the removal of water that exists on the surface of α -Fe₂O₃. Differential thermal analysis (DTA) revealed an exothermic peak at 54.4 °C, indicating a heat release during this stage.

In the second stage, one endothermic peak was observed at 298.7 °C with a 1.02% weight loss. This indicates the decomposition of the biomolecules from the plant extract used in the synthesis process. The temperature at which the weight loss became constant was used as the calcination temperature for the synthesized α -Fe₂O₃ NPs. In the final step, there were no signs of any specific weight losses throughout the range of 587.7–800 °C, and exothermic peaks were observed in the DTA curve at 534.1 °C. The calcination temperature is the temperature above which the weight loss of the sample is kept constant. After 587.7°C, the curve becomes parallel to the temperature axis, indicating the high stability of α -Fe₂O₃ NPs as shown in Figure 19 (a) (Lassoued et al., 2020).

The thermal behavior of as-prepared ZnO nanocrystals was investigated using DTA and TGA, as shown in Figure 19 (b). The TGA curve displayed two weight losses. The first weight loss step occurred gradually between [121.1-134.7 °C], with a mass loss of 0.52%, attributed to the removal of water from the surface of ZnO. DTA analysis indicated one exothermic peak at 45.2 °C. The second weight loss occurred in the range of (134.7-438 °C) and with a loss of 1.83%, which may be due to the decomposition of residual compounds. The TGA curve showed a flat ground above 438°C, indicating the formation of ZnO NPs as a decomposition product. The DTA curve displayed endothermic peaks at 53 °C and 370.5 °C. Additionally, an exothermic peak was observed in the range of 582.1 °C, which appeared due to thermal lattice vibrations. There was no further weight loss above 438.2 °C, indicating the optimum annealing temperature for this study is 438.2 °C. The results from the TGA and DTA analyses provide important insights into the thermal behavior and properties of ZnO nanocrystals, which can be used to

optimize the synthesis process and improve the performance of the material for various applications (Raji & Gopchandran, 2017).

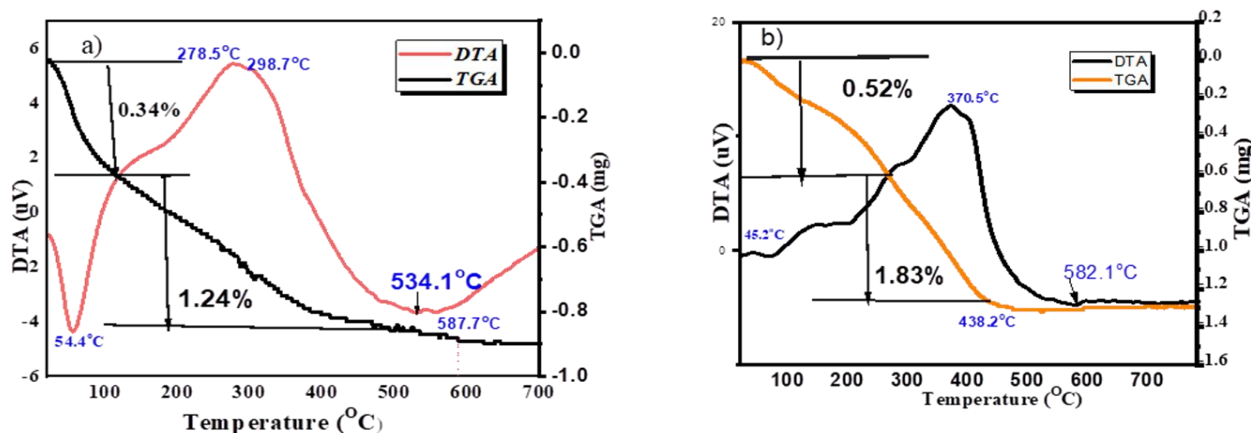


Figure 15. TGA/DTA graph of (a) α -Fe₂O₃ NPs and (b) ZnO NPs.

TGA and DTA are powerful thermal analysis techniques that can be used to uncover the fascinating properties of bentonite. These techniques can reveal a wealth of information about the water content, thermal stability, decomposition, and phase transitions of bentonite. Three weight losses in the TGA curve, each corresponding to a different aspect of the clay's behavior are observed. The first weight loss of 0.76% occurred between 40 °C and 150 °C and was due to physically adsorbed water. The second weight loss of 2.21% occurred between 150 °C and 321 °C and was assigned to the elimination of water coordinated to the interlayer cations in cation-exchanged bentonite. At the same time, the DTA curve revealed even more fascinating results. It showed an endothermic peak at 440 °C, which corresponded to the dehydroxylation of the clay mineral. Additionally, three endothermic and one exothermic peak were observed at 289.1 °C, 349.4 °C, 440 °C, and 80.3 °C, respectively (Atkovska et al., 2016).

Finally, the weight loss of 4.91% observed in the TGA curve between 321 °C and 700 °C was attributed to the structural dehydration of neighboring hydroxyl groups as indicated in Figure 20 (a). To improve bentonite purity, removed the clay residues by calcining at over 550 °C (Donoso et al., 2010). Bentonite with ZnO NPs showed a weight loss of 0.24% at (32-93 °C) was due to physically adsorbed water, another weight loss of 1.04% at (93-645 °C) due to lattice water and dehydroxylation of the clay mineral, and two exothermic processes at 70.8 and 221.8 and one endothermic at 532 °C were observed during the heating process finally pure composite were obtained above 646 °C. Bentonite with ZnO NPs showed a weight loss of 0.24% at (32-93 °C)

due to physically adsorbed water, another weight loss of 1.04% at (93-645 °C) due to lattice water and dehydroxylation of the clay mineral, and at 70.8 and 221.8 °C which represent the exothermic reaction and 532.1 °C it is endothermic process finally pure composite were obtained at 700 °C as indicated in Figure 20 (b).

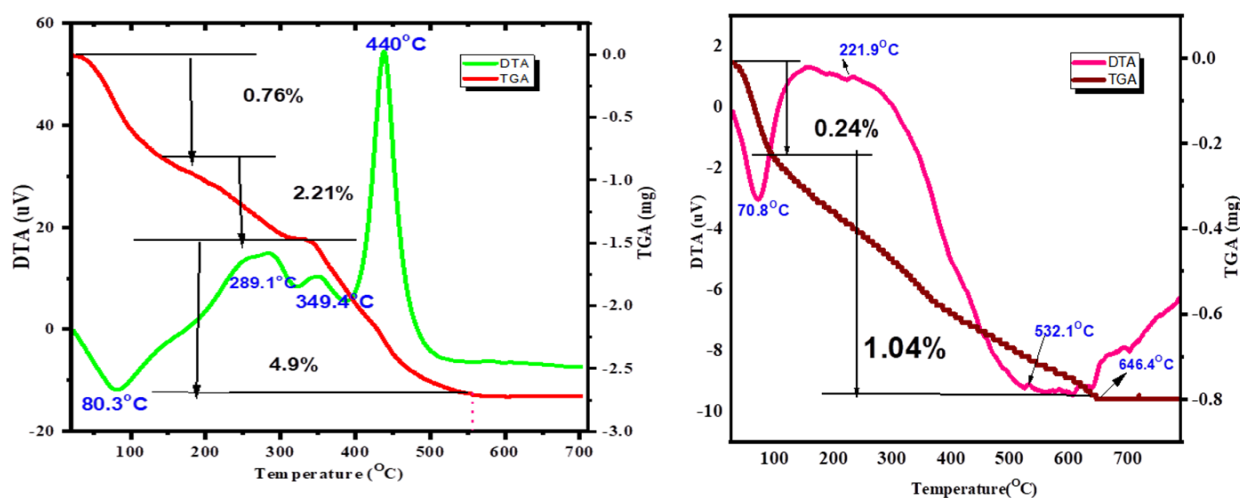


Figure 16. TGA/DTA graph of a) Activated Bentonite and b) ZnO/Bentonite

Similarly, the thermal stability of α -Fe₂O₃/bentonite is demonstrated in Figure 21(a) which displays the TGA-DTA analyses. The thermograph shows three distinct decomposition regions. The first weight loss of 0.37 wt% occurred at a lower temperature range of (44-67 °C) due to the release of weakly bound water molecules and dehydroxylation of the clay material. The second weight loss of 1.5 wt% and 2.15 wt% occurred between 67 and 215°C and from 215 to 574 °C, respectively. The weight loss between 215 and 574 °C was caused by the presence of volatile organic compounds in the produced sample, the condensation of the sinol group (Si-O) compound present in the bentonite, and the decomposition of chemical components, indicating a crystalline phase transformation and the temperatures above 583 °C, the weight of the synthetic α -Fe₂O₃/bentonite remained constant, indicating its high purity and stability at that temperature. The decomposition of physically and chemically adsorbed water, as well as the breakdown of chemical components, was observed in three distinct regions. DTA demonstrated an endothermic peak at 277.9 and 289.7 °C, and an exothermic peak between 62.7 and 547.8°C, followed by a stable curve at 583.4°C, highlighting the remarkable stability of α -Fe₂O₃ with bentonite. Notably, the TGA curve did not show a corresponding signal. These findings confirm the crystallization and phase transition of α -Fe₂O₃ with bentonite, as reported by (Tao et al., 2009).

Finally, Figure 21(b) illustrates how ZnO/ α -Fe₂O₃/Bentonite behaves during the TGA-DTA analysis. The TGA curve shows that the majority of weight loss occurred below 565.7 °C. The first weight loss of 0.4% up to 95°C was due to the removal of adsorbed water and moisture, supported by endothermic peaks observed at 165.9, 263, and 517.1°C in the DTA curve. The second weight loss of 1.3% up to 327 °C was due to the evaporation of crystalline bonded water, and crystallization of ZnO/ α -Fe₂O₃/Bentonite. The third weight loss of 2.3% at 327-749 °C was attributed to the decomposition of some organic compounds in the phytochemicals and crystallization of ZnO/ α -Fe₂O₃/Bentonite. No significant weight loss was observed above 565.7 °C, indicating the complete formation of ZnO/ α -Fe₂O₃/Bentonite crystals up to that temperature. The calcination temperature of 565.7 °C was chosen to produce a composite phase of pure ZnO/ α -Fe₂O₃/Bentonite NCs, as reported by (Ajala et al., 2020; Atkovska et al., 2016; Dejen et al., 2020).

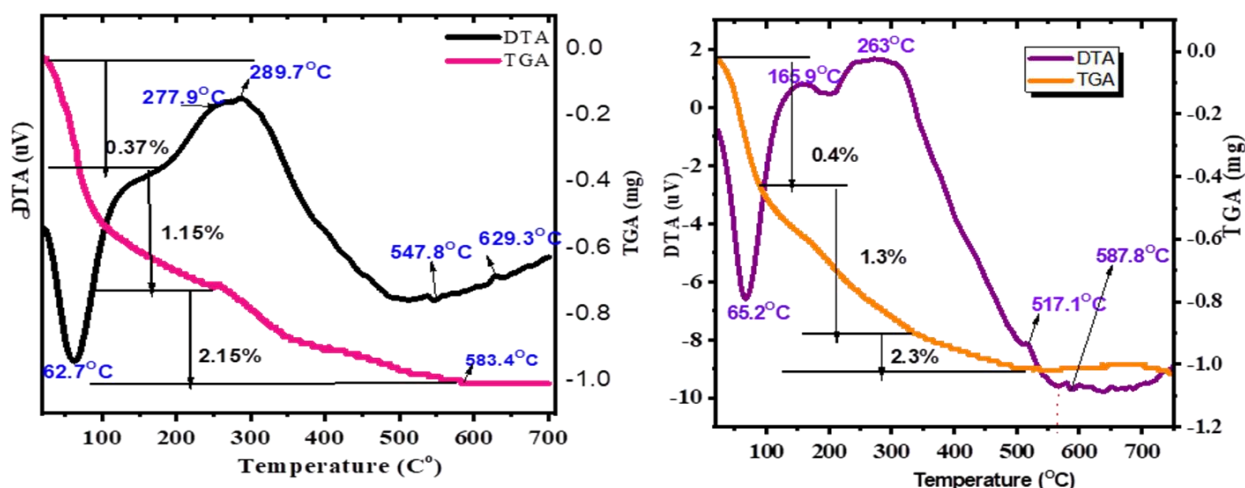


Figure 17. TGA/DTA graph of a) α -Fe₂O₃/Bentonite and b) ZnO/ α -Fe₂O₃/Bentonite

4.7 Photoluminescence spectroscopy analysis

Photoluminescence (PL) spectroscopy is crucial for determining photo generated electron and hole pairs are separated in semiconductor materials. As depicted in Figure 22, the intensity of the PL band decreases when ZnO NPS are decorated with α -Fe₂O₃ and bentonite, indicating a higher separation efficiency of photo-induced charge carriers compared to the bare sample, as reported by (da Trindade et al., 2020). Higher intensity indicates a faster rate of recombination, while lower intensity indicates a larger amount of transferred or trapped electrons. The 1:1:1

ZnO/ α -Fe₂O₃/Bentonite NCs exhibit much lower emission intensity than others, indicating that the recombination of photo-generated electron-hole pairs was effectively inhibited, as reported by Rufus et al. (2016). The 1:1:1 ZnO/ α -Fe₂O₃/Bentonite NCs sample has a lower electron-hole pair recombination rate. Therefore, higher the intensity of PL the higher the rate of recombination rate which leads to lower the efficiency of the composite activity (Jan et al., 2019). Recombination and efficiency are inversely related. The efficiency order should be single < binary < ternary composite as shown in Figure 22.

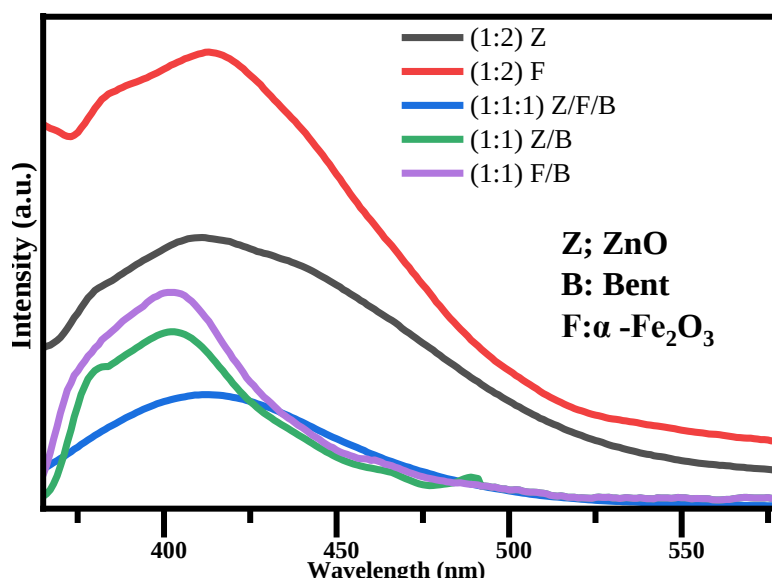


Figure 18. Photoluminescence spectra of (1:2) ZnO NPs, (1:2) α -Fe₂O₃ NPs, (1:1) ZnO/Bentonite NCs, (1:1) α -Fe₂O₃/Bentonite NCs, (1:1:1) and (1:1:1) ZnO/ α -Fe₂O₃ /Bentonite NCs.

4.8 Fourier transforms infrared studies

Fourier transform infrared (FT-IR) measurements were conducted to identify the primary functional groups in the plant leaf extract, as well as in the synthesized α -Fe₂O₃ NPs, ZnO NPs, α -Fe₂O₃/Bentonite, ZnO/Bentonite, and α -Fe₂O₃/ZnO/Bentonite NCs that were synthesized using plant leaf extract. This was done to investigate the potential role of the plant leaves extract in the synthesis and stabilization of NPs and NCs.

Table 4. FT-IR Profile of the *H. abyssinica* plant leaves extract

Peak values (cm ⁻¹)	Functional group	Class
3400	-OH	Alcohol
2930	-C-H (Stretch)	Alkane
2845	C-H	Alkane
2260	C≡N	Nitrile
2072	C≡C stretching	Alkyne
1628	C=C	Alkene(conjugated)
1359	C-O	Alcohol
1010	C-O (stretch)	Alcohols

The peak observed at 3400 cm⁻¹ is typical of -OH (alcohol) while 2921 cm⁻¹ and 2847 cm⁻¹ can be assigned to C-H (stretch) for alkane. The peak at 2316 cm⁻¹ indicates the presence of a nitrile group (Zewudie et al., 2023). Also, the peak at 1619 cm⁻¹ (C=C) is that of conjugated alkene. The signal at 1323 (C-O) is that of alcohol and 1010 cm⁻¹ (C-O stretch) is typical of alcohols also (Table 8 and Figure 23 (a)). The FTIR analysis revealed the presence of two basic functional groups; C=O and O-H which indicate the presence of alcohol, phenol, or esters. The O-H (Alcohol/ Phenol) group was observed in the leaf extract with wavelengths of 3400 cm⁻¹. The peaks between the ranges of 1000 – 1500 cm⁻¹ are present in the extract due to the presence of the C-O bond. This band may either be alcohol, ester, ether, or anhydride. The presence of secondary amine was also detected at 1628 cm⁻¹ for the extract. Tannin and flavonoids are simple phenolic compounds that serve as a defense against pathogens (S. Kumar et al., 2020).

The FT-IR result presented in Figure 23 (b) provides information on the fundamental vibrational modes of the constituent units of bentonite clays. The spectra show four main absorption regions: 3600–2900 cm⁻¹, 2900–2850 cm⁻¹, 1630–1440 cm⁻¹, and 1030–462 cm⁻¹. Activated bentonite exhibits the characteristic montmorillonite absorption band at 3620 cm⁻¹, indicating Al-OH-Al along with the Al-Mg-OH stretching vibrations. This indicates the stretching vibration of ⁻OH of water coordinated to octahedral Al³⁺ cations. The activation of bentonite with Na₂CO₃ has altered the peaks of the bands associated with absorbed water at 3435 cm⁻¹ (H-O-H stretching) and decreased the peaks of the bands associated with absorbed water at 1630 cm⁻¹ (H-O-H bending). The changes in the intensities of these bands are explained by the loss of water molecules when pure bentonite is transformed into activated bentonite. The absorption band observed at 1020 cm⁻¹ is indicative of montmorillonite strong Si-O stretching vibration, which

provides strong evidence for a silicate structure (Zewudie et al., 2023). Moreover, the intensity of the band at 1020 cm^{-1} increased due to the formation of three-dimensional networks of amorphous Si-O-Si units. The band observed at 690 cm^{-1} corresponds to coupled Al-O and Si-O out-of-plane vibrations (A. Kumar & Lingfa, 2020).

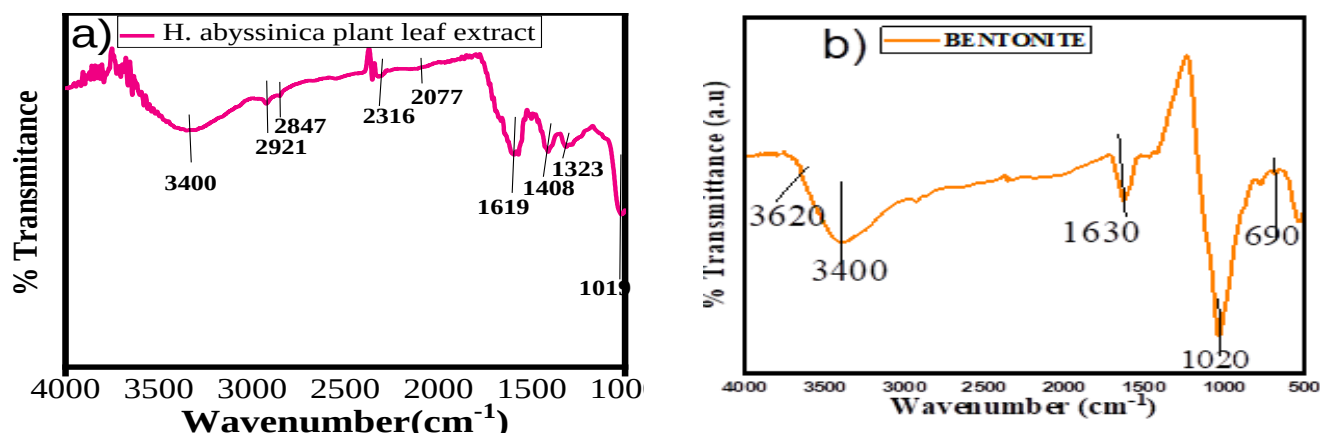


Figure 19. FT-IR result of a) *H. abyssinica* plant leaf and b) Activated bentonite.

Also, Figure 24(a) displays the FT-IR absorption bands of the synthesized ZnO NPs (calcined at 450°C for 2 hour) in the range of $500 - 4000\text{ cm}^{-1}$. The bands are located at 3405 cm^{-1} , 2350 cm^{-1} , 2169 cm^{-1} , 1989 cm^{-1} , 1439 cm^{-1} , 1000 cm^{-1} , 881 cm^{-1} , and 526 cm^{-1} . The broad peaks around 3405 cm^{-1} correspond to the H-bonded or OH stretch bond vibration of polyphenols and flavonoids, and they are also due to water adsorption on the surface of ZnO nanoparticles. The peaks at 2350 cm^{-1} correspond to N-H stretching or the C=O stretching vibrations (Awwad et al., 2014). The band observed at 2169 cm^{-1} is due to C=C stretching (Dobrucka & Długaszewska, 2016), while C=C=C stretching was observable at 1989 cm^{-1} . The band at 1439 cm^{-1} was attributed to the C-C stretching of the aromatic ring (Suresh et al., 2018). The band at 1000 cm^{-1} is assigned to the absorption of C-O (stretch), and the bands at 881 cm^{-1} are due to the C=C bending of alkene. The peak in the region between 400 and 600 cm^{-1} is attributed to Zn-O (Yuvakkumar et al., 2015).

Similarly, FTIR analysis (Figure 24(b)) provided information about the functional groups of biomolecules responsible for reducing and capping the $\alpha\text{-Fe}_2\text{O}_3$ nanoparticles. The spectrum showed prominent bands at 602 cm^{-1} , 545 cm^{-1} , and 465 cm^{-1} , which can be attributed to Fe-O vibrational modes. Additionally, the peak at 993 cm^{-1} corresponds to the C-H bend of alkene, while the bands at 1388 cm^{-1} and 1635 cm^{-1} are assigned to the C=O stretching of alcoholic

derivatives. The presence of the FeO_6 octahedron in the samples was confirmed by the absorption bands at 549 cm^{-1} , which were attributed to the stretching of the Fe-O bond (Efficiencias et al., 2022). The FTIR spectrum of the hematite NPs exhibited a peak in the amide region (1509 cm^{-1}), which is characteristic of proteins and enzymes responsible for the reduction of the metal ions from the precursor. Furthermore, O-H bending vibrational modes resulted in a band at 1670 cm^{-1} , and the band at 668 cm^{-1} was due to aromatic C-H out-of-plane bending vibrations. The absence of bands at 1408 cm^{-1} and 1323 cm^{-1} of the leaf extract in the FTIR spectrum of hematite NPs suggests the involvement of polyphenolic compounds in reduction and stabilization (Rufus et al., 2017). This strong band may also be due to the presence of the C=O group of the extracted plant capping $\alpha\text{-Fe}_2\text{O}_3$ NPs (Rufus et al., 2017).

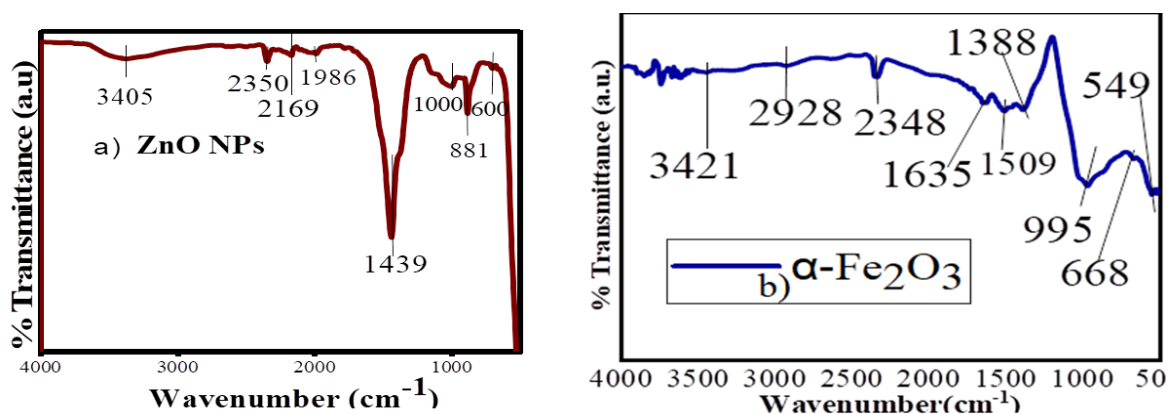


Figure 20. FTIR result of the ZnO NPs and $\alpha\text{-Fe}_2\text{O}_3$.

Finally, FT-IR spectroscopy was also used to characterize the functional groups of $\alpha\text{-Fe}_2\text{O}_3$ /Bentonite composites, ZnO/bentonite composites, and $\alpha\text{-Fe}_2\text{O}_3$ /ZnO/Bentonite composites in the range $500 - 4000\text{ cm}^{-1}$. This technique is commonly used to investigate the structure, bonding, and reactivity of clay minerals and metal oxides. The NCs exhibited bands at around $3490 - 3590\text{ cm}^{-1}$, which can be assigned as the stretching vibration absorption peaks of O-H. The bands at $1576 - 1610\text{ cm}^{-1}$ were attributed to C=O and C=C bending mode of vibrations, while the band at $1392 - 1415\text{ cm}^{-1}$ suggested the presence of C-C stretching vibration (Chakraborty et al., 2019; Kassem, 2015). Furthermore, the absorption band observed at 1020 cm^{-1} in the spectra is indicative of montmorillonite strong Si-O stretching vibration, which provides strong evidence for a silicate structure.

The band at 672 cm^{-1} and 689 cm^{-1} corresponded to a couple of Al-O and Si-O out-of-plane vibrations, while the peak at 1020 cm^{-1} was also observed for the bending vibration of Si-O-Zn and Si-O-Fe. Additionally, the peak at 789 cm^{-1} to 770 cm^{-1} and 467 cm^{-1} was assigned to the bending vibration of the Si-O-Si bond and the lattice vibration of Si-O and Al-O, respectively (A. Kumar & Lingfa, 2020). The absorption peaks at 520 cm^{-1} , 533 cm^{-1} , and 420 cm^{-1} in $\alpha\text{-Fe}_2\text{O}_3$ /Bentonite, ZnO/bentonite, and ZnO/ $\alpha\text{-Fe}_2\text{O}_3$ /Bentonite NCs were assigned to the Zn-O and Fe-O bond, similar to another study. Furthermore, the intensity of the stretching band observed at 3627 cm^{-1} for activated bentonite by sodium carbonate (Al-OH-Al along with the Al-Mg-OH stretching vibrations) indicated the involvement of plant extract in the formation of nanocomposites (Yang et al., 2021). The symmetric peak observed at 420 cm^{-1} and 520 cm^{-1} corresponded to the characteristic ZnO NPs and $\alpha\text{-Fe}_2\text{O}_3$, respectively, and was consistent with Si-O-Al. The band present in the range of $1402 - 1624\text{ cm}^{-1}$ in both composites indicated the successful incorporation of the leaf extract and precursor with the produced nano-clay. These findings were confirmed by the broad peak observed at $3438\text{ cm}^{-1} - 3627\text{ cm}^{-1}$ in the $\alpha\text{-Fe}_2\text{O}_3$ /Bent NC, which increased with higher amounts of NPs. This peak was attributed to the van der Waals interactions between the hydroxyl groups of MMT layers and the partial positive charge on the surface of ZnO NPs and $\alpha\text{-Fe}_2\text{O}_3$ NPs. These results suggest a certain interaction between the clay and the NPs as indicated in Figure 25.

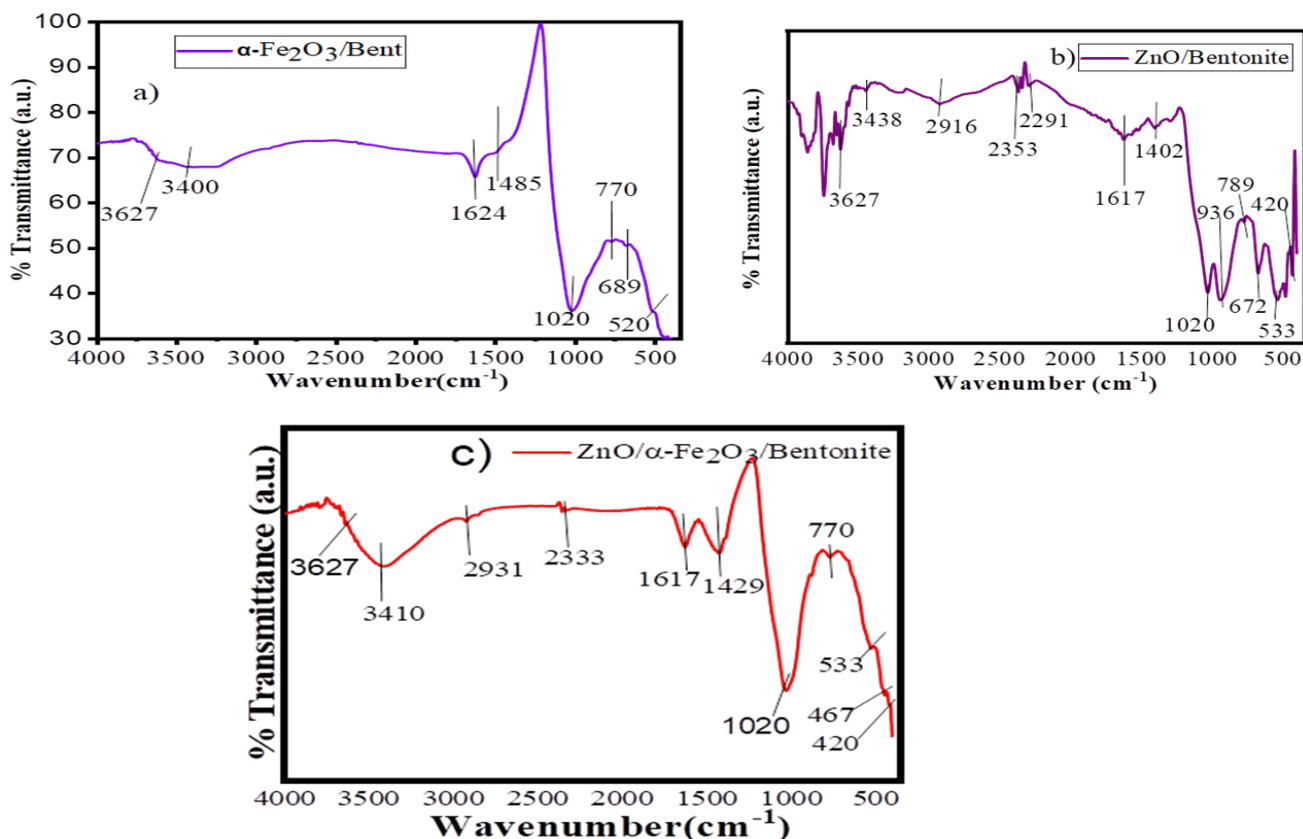


Figure 21. FT-IR result of the ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite.

4.9 Antibacterial Activity test of the synthesized, ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite Nanocomposite

Antibacterial activity of the synthesized NPs and NCs against, Gram-positive (*Staphylococcus aureus* ATCC25923) and Gram-negative (*Escherichia coli* ATCC25923) bacteria using the disc diffusion method. The study employed different volume ratios, with Gentamicin (25 μ g) antibiotics as a positive control and DMSO as a negative control, which had no activity on either bacterial strain and was used as a solvent in sample serial dilution. The NPs and NCs synthesized using various ratios of bentonite, metal oxide precursor, and *H. Abyssinica* plant leaf extract, including α -Fe₂O₃, ZnO, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite, exhibited promising antibacterial properties.

The antibacterial mechanisms of the NPs against bacteria include oxidative stress induction, metal ion release, or non-oxidative mechanisms. Understanding the antibacterial mechanisms of NPs is crucial for developing more effective antimicrobial materials to combat bacterial

infections (Azam et al., 2012). Figures 26 - 33 shows images of the antibacterial test results for the synthesized substances. The results reveal that the synthesized NPs and NCs exhibit antibacterial properties, as evidenced by the presence of inhibitory zones. It was observed that Na-bentonite and purified bentonite did not exhibit any antibacterial activity. While the antibacterial effects were more pronounced on Gram-positive bacteria, specifically *S. aureus*, the plant powder demonstrated some effect as indicated by the inhibitory zone. Bentonite can have varying antibacterial activity depending on its mineral composition, which can differ depending on where it comes from and how it is processed. Generally, bentonite that contains higher amounts of certain minerals like aluminum, iron, and magnesium tends to exhibit stronger antibacterial properties. In the case of sodium bentonite, it may not show antibacterial properties because it has a lower concentration of these antibacterial minerals compared to other types of bentonite (Figure 26). This lower concentration of minerals may inhibit the ability of sodium bentonite to effectively target and kill bacteria (Zewudie et al., 2023).

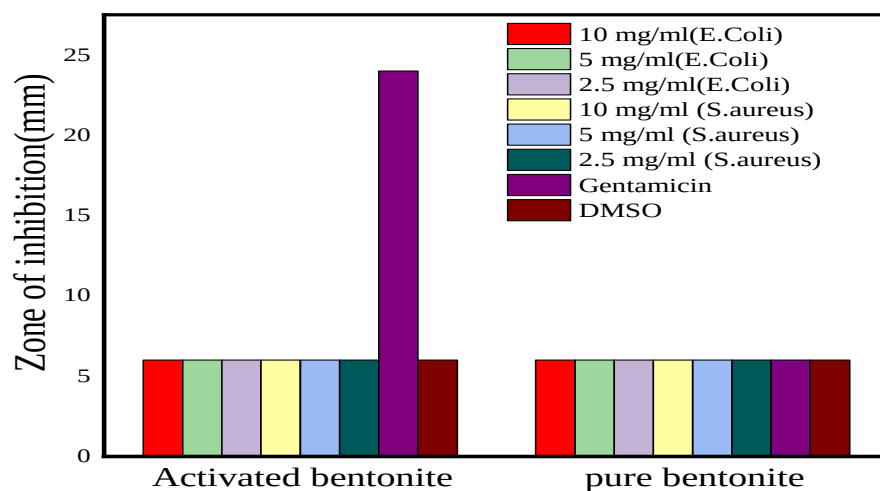


Figure 22. Antibacterial activities of activated bentonite and pure bentonite

Plant leaves extracted has natural antibacterial properties that stem from a variety of chemical compounds present in the powder, such as phenolic and derivative compounds, esters of weak and fatty acids, and flavonoids. These compounds serve as a defense mechanism against bacteria and fungi for plant (Platzer et al., 2022). These chemical components can target multiple sites within bacterial cells, leading to their destruction. However, the effectiveness of plant leaves extracted against different types of bacteria can vary. The graph tells us as *S.*

aureus is more resistant than *E. coli*. This is because *E. coli* has an additional outer membrane made up of lipopolysaccharides, which creates an extra layer of resistance called periplasm.

In contrast, Gram-positive bacteria like *S. aureus* have a thick layer of peptidoglycan in their cell walls, which can make them more susceptible to the chemical components of plant powder. The different sensitivities of Gram positive and Gram-negative bacteria could be explained in accordance with the morphological differences between these microorganisms, due to different polarities of their cell membrane. The membrane of *S. aureus* has a lower negative charge than that of *E. coli*. This would allow a higher level of penetration of negatively charged free radicals such as, superoxide radical anions and peroxide ions, causing *S. aureus* damage and cell death at lower concentrations than those required to damage *E. coli* (Gray & Wenzel, 2020). Nonetheless, the chemical components in plant powder can still affect multiple targets within bacterial cells, making them a promising source of natural antibacterial agents (Figure27).

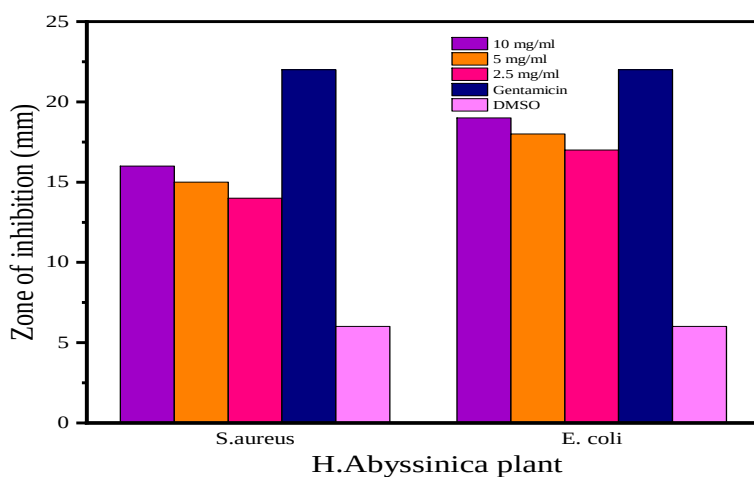


Figure 23. Antibacterial activities of *H. abyssinica* plant leaves extract

The antibacterial efficacy of α -Fe₂O₃ NPs synthesized in the study was investigated against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. To assess the antibacterial efficacy of α -Fe₂O₃ nanoparticles at various concentrations (10, 5, and 2.5 mg/mL), the viable disk diffusion method was used for (1:1 and 1:2) α -Fe₂O₃ NPs. The growth of the bacteria strains exposed to different concentrations of α -Fe₂O₃ NPs was analyzed, and the results are presented in Figure 28. The findings revealed that α -Fe₂O₃ NPs had a significant impact on the growth of both *E. coli* and *S.*

aureus, with the maximum inhibition zone being recorded for *S. aureus* at 100 $\mu\text{g/mL}$ concentration of $\alpha\text{-Fe}_2\text{O}_3$ (1:1) ratio, measuring $10\pm0.3\text{mm}$, $9\pm0.3\text{mm}$, and $8\pm0.3\text{mm}$. Similarly, at the same concentration, $\alpha\text{-Fe}_2\text{O}_3$ (1:2) NPs demonstrated excellent antibacterial activity against both strains, with the zone of inhibition measuring $11\pm0.3\text{mm}$, $10\pm0.3\text{mm}$, and $8\pm0.3\text{mm}$ for *S. aureus* and $9\pm0.3\text{mm}$, $8\pm0.3\text{mm}$, and $7\pm0.3\text{mm}$ for *E. coli*. Moreover, the $\alpha\text{-Fe}_2\text{O}_3$ NPs synthesized using the (1:2) ratio exhibited higher antibacterial activity against all strains compared to those synthesized using the (1:1) ratio for all concentrations due to the higher concentration of extracted plant. These findings demonstrate the potential use of $\alpha\text{-Fe}_2\text{O}_3$ NPs as effective antibacterial agents against *E. coli* and *S. aureus* (Ozdal & Gurkok, 2022).

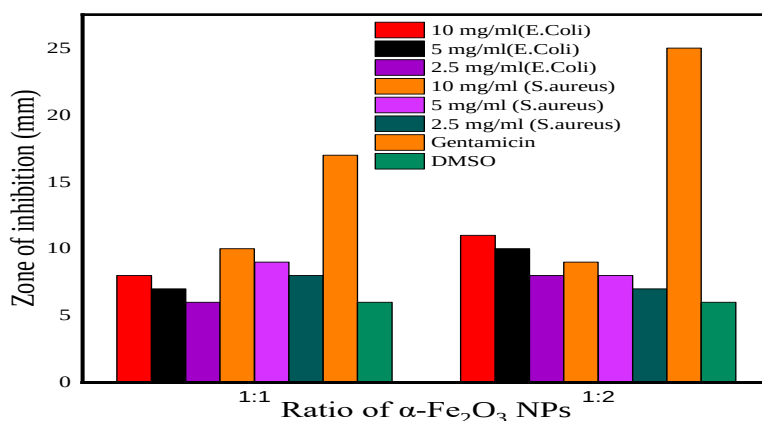


Figure 24. Antibacterial activities of (1:1 $\alpha\text{-Fe}_2\text{O}_3$ NPs) and (1:2 $\alpha\text{-Fe}_2\text{O}_3$)

Figures 29 presents the results of the antibacterial activity of ZnO NPs which was synthesized in three different volume ratios, namely 1:1, 1:2, and 2:1. The findings indicate that the 1:2 ratio of the synthesized ZnO NPs showed the strongest antibacterial efficacy compared to the other ratios. This is due to the smaller average crystalline size and the presence of higher concentrations of bioactive compounds found in the leaf extract, which leads to higher ROS production. The results showed that the tested pathogenic bacteria were significantly inhibited by ZnO NPs with remarkable antimicrobial activity. The antibacterial activity was found to be higher for ZnO NPs with a ratio of 1:2 compared to those with ratios of 1:1 and 2:1. This observation was consistent with previously reported studies (Mendes et al., 2022), which found that the zone diameter increased as the size of ZnO NPs decreased, likely due to the bactericidal activity of ZnO. These suggest that ZnO nanoparticles have the ability to enhance the

antimicrobial activity of zinc ions, and that smaller zinc ion-containing ZnO nanoparticles may have greater bactericidal effects.

In the 1:1 ratio, ZnO NPs exhibited good performance against both *S. aureus* and *E. coli*, with a maximum zone of inhibition of 12 ± 0.3 mm at 10 mg/mL concentration against *S. aureus*, and a range of zone of inhibition between 7 - 12 mm. The bioactive compound in this ratio is found in equal proportion with the precursor salt, allowing for interaction with the surface of the bacteria cell wall and penetration. The 2:1 ratio showed weaker antibacterial activity than the other ratios, with a zone of inhibition of 8 ± 0.3 mm at 10 mg/mL concentration against *S. aureus* and 7 ± 0.3 mm at 10 mg/mL concentration against *E. coli*. This may be due to the larger average crystalline size of the formed ZnO NPs from XRD results compared to the other ratios, which affects its antibacterial efficacy. Overall, the findings suggest that biosynthesized ZnO NPs have potential as antibacterial agents, with the 1: 2 ratios showing the strongest activity against both bacteria strains.

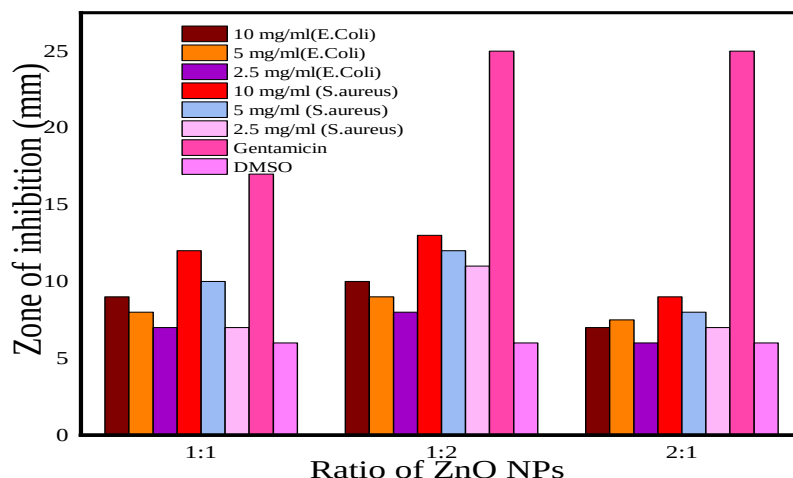


Figure 25. Antibacterial efficacy for ZnO NPs at ratios of (1:2), (2:1), and (1:1).

The composites of α -Fe₂O₃/Bentonite in different ratios exhibit promising antibacterial properties as depicted in Figure 30, suggesting that this composite has higher antibacterial activity as compared to individual NPs. The inhibitory zone results of (1:1) α -Fe₂O₃/Bentonite against *E. coli* ATCC 25922 were 14 ± 0 mm at 10 mg/mL, 13.3 ± 0.6 mm at 5 mg/mL, and 12 ± 0.6 mm, while against *S. aureus* ATCC 25923, they were 15 ± 0 mm at 10 mg/mL, 12 ± 0.3 mm at 5 mg/mL, and 8 ± 0.3 mm. The equal mixing of bentonite and α -Fe₂O₃ in the (1:1) ratio

enhances their individual antibacterial properties, providing a larger surface area for bacterial adsorption and inhibiting the growth and multiplication of a wide range of bacteria. The (1:2) ratio of α -Fe₂O₃/Bentonite composite also showed good antibacterial activity as compared to (2:1) with inhibitory zone results of 10 ± 0 mm at 10 mg/mL, 9 ± 0.6 mm at 5 mg/mL, and 8 ± 0.6 mm against *E. coli* ATCC 25922, and 10 ± 0 mm at 10 mg/mL, 9 ± 0.3 mm at 5 mg/mL, and 8 ± 0.3 mm against *S. aureus* ATCC 25923. In contrast, the (2:1) ratio of Fe₂O₃/Bentonite composite exhibited weaker antibacterial activity, with inhibitory zone results of 8 ± 0 mm at 10 mg/mL, 7 ± 0.6 mm at 5 mg/mL, and 6 ± 0.6 mm against *E. coli* ATCC 25922, and 9 ± 0 mm at 10 mg/mL, 8.5 ± 0.3 mm at 5 mg/mL, and 8 ± 0.3 mm against *S. aureus* ATCC 25923. These results suggest that α -Fe₂O₃ /Bentonite composites in the (1:1) ratios have the potential for use as effective antibacterial agents due to the synergistic effect between bentonite clay and α -Fe₂O₃.

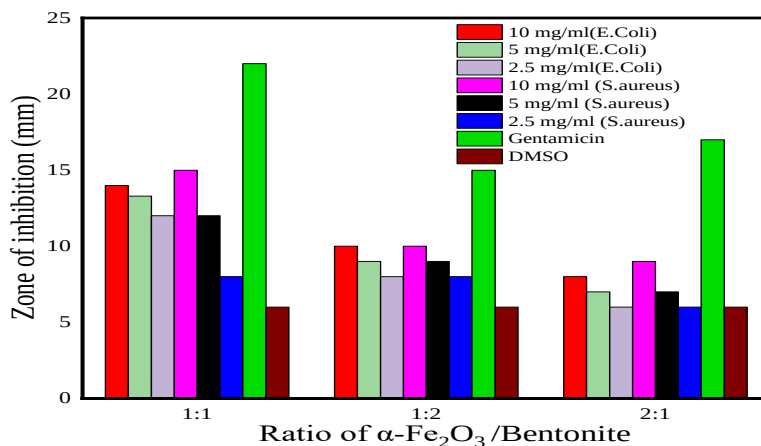


Figure 26. Antibacterial activities of (1:1), (1:2), and (2:1) of α -Fe₂O₃ /Bentonite.

The antibacterial activity of ZnO/bentonite NCs in different ratios (1:1, 1:2, 2:1) was evaluated, with results indicating that (1:1) ZnO/bentonite NCs exhibit stronger antibacterial activity compared to the other ratios. These results are presented in Figure 31. The (1:1) ZnO/bentonite NCs showed the highest antibacterial activity, with the largest inhibitory zones against both bacterial strains tested. The inhibitory zone results of (1:1), (1:2), and (2:1) ZnO/bentonite NCs against *E.coli* were 11 ± 0.3 mm at 10 mg/mL, 11.23 ± 0.6 mm at 5 mg/mL, and 10 ± 0.6 mm at 2.5 mg/mL, 10 ± 0 mm at 10 mg/mL, 9.5 ± 0.3 mm at 5 mg/mL, and 8 ± 0.6 mm at 2.5 mg/mL and 9 ± 0 mm at 10 mg/mL, 8 ± 0.6 mm at 5 mg/mL, and 7 ± 0.6 mm at

2.5mg/mL respectively. Similarly, the inhibitory zone results against *S. aureus* were 14 ± 0.3 mm at 10 mg/mL, 13 ± 0.6 mm at 5 mg/mL, and 11 ± 0.6 mm at 2.5 mg/mL, 8.5 ± 0.3 mm at 10 mg/mL, 7.5 ± 0.6 mm at 5 mg/mL, and 6 ± 0.6 mm at 2.5 mg/mL, and 10 ± 0 mm at 10 mg/mL, 8 ± 0.6 mm at 5 mg/mL, and 7.5 ± 0.6 mm at 2.5 mg/mL for (1:1), (1:2), and (2:1) ZnO/bentonite NCs, respectively, which shown in Appendix. Overall, the results suggest that (1:1) ZnO/bentonite NCs have the greatest potential for use as an effective antibacterial agent against both *E. coli* and *S. aureus*.

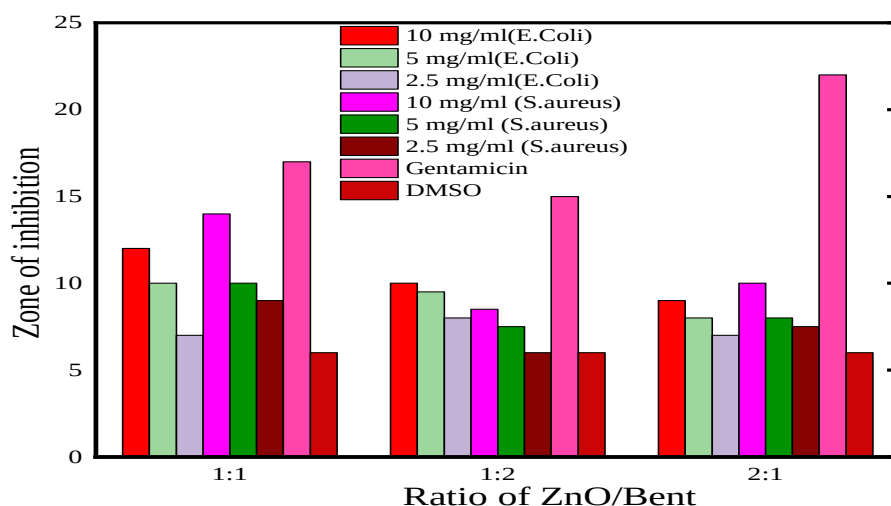


Figure 27. Antibacterial activities of (1:1), (2:1), and (1:2) ZnO/bentonite.

Both α -Fe₂O₃ and ZnO possess bactericidal properties, and their NPs forms have been found to have more significant antibacterial effects on a broad range of microorganisms compared to their bulk material. At the nano-scale, their small size allows for better penetration and interaction with bacteria, leading to the disruption of the cell membrane and increased effectiveness as bactericidal material. Furthermore, a combination of the two composites (α -Fe₂O₃ /ZnO/Bentonite) has been shown to possess antibacterial activity, with greater efficacy observed against Gram-positive *S. aureus* ATCC 25923 compared to Gram-negative *E. coli* ATCC 25922. The inhibitory zone results of (1:1:1), (2:2:1), and (1:1:2) α -Fe₂O₃ /ZnO/Bentonite NCs were 13 ± 0.3 mm at 10 mg/mL, 12 ± 0.6 mm at 5 mg/mL, and 11 ± 0.6 mm at 2.5 mg/mL, 10 ± 0 mm at 10 mg/mL, 9.5 ± 0.3 at 5 mg/mL, and 8 ± 0.6 mm, and 9 ± 0 mm at 10 mg/mL, 8 ± 0.6 mm at 5 mg/mL, and 7.5 ± 0.6 mm at 2.5 mg/mL against *E. coli*. Similarly, the inhibitory zone results against *S. aureus* were 16 ± 0.3 mm at 10 mg/mL, 14 ± 0.6 mm at 5 mg/mL, and 13 ± 0.6 mm, 10 ± 0.3 mm at

11mg/mL, 10 ± 0.6 mm at 5 mg/mL, and 9 ± 0.6 mm, and 10 ± 0 mm at 10 mg/mL, 8 ± 0.6 mm at 5 mg/mL, and 7.5 ± 0.6 mm at 2.5 mg/mL. The (1:1:1) ratio of α -Fe₂O₃/ZnO/Bentonite NCs showed higher antibacterial activity, which may be due to the more effective modification of the surface of α -Fe₂O₃ and ZnO by bentonite as indicated in Figure 32.

This results in the formation of a more stable composite, leading to better efficiency compared to other ratios and also sodium bentonite high cation exchange capacity that has the potential to interfere with cations like Zn(II) and Fe (III) to inhibit or neutralize its antibacterial activity. Therefore, Antibacterial activity of the composites can be attributed to chemical and physical properties of the nanoparticles together with the large surface area of the clay. This means that a large number of nanoparticles with smaller size were highly dispersed per unit area of the bentonite surfaces, thus, maximizing antibacterial activity. In such combinations, nanoparticles are largely attached on bentonite surfaces which ensure uniform dispersion, as unsupported nanoparticles tend to agglomerate and hence reducing their final properties(V.A.R. Baro et al., 2022). The antibacterial mechanisms of NPs against bacteria include oxidative stress induction, metal ion release, or non-oxidative mechanisms. Understanding the antibacterial mechanisms of NPs is crucial for developing more effective antimicrobial materials to combat bacterial infections.

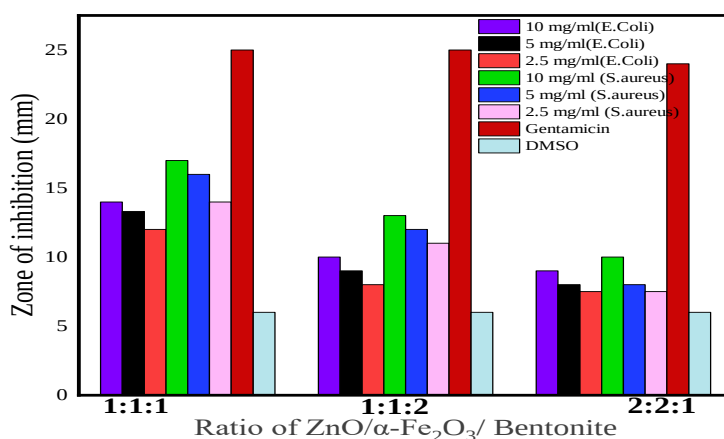


Figure 28. Antibacterial activities of (1:1:1), (2:2:1) and (1:1:2) of ZnO/ α -Fe₂O₃/Bentonite

To investigate the statistical significance of the antimicrobial activity data P-values were calculated for all tests. The P value is defined as the probability under the assumption of no effect or no difference (null hypothesis), of obtaining a result equal to or more extreme than what

was observed. The P stands for probability and measures how likely it is that any observed difference between groups is due to chance. As p-values less than 0.05 ($P < 0.05$) in all data, the data was statistically significant and acceptable. Being a probability, P can take any value between 0, and Values close to 0 indicate that the observed difference is unlikely to be due to chance, whereas a P value close to 1 suggests no difference between the groups other than due to chance.

4.9.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of (1:1:1) α -Fe₂O₃/ZnO/Bentonite nanocomposites.

The Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) of ZnO/ α -Fe₂O₃/ Bentonite NCs were determined to assess their antibacterial activity. The MIC is the lowest concentration of a substance that inhibits the visible growth of bacteria, while the MBC is the lowest concentration of a substance that kills the bacteria. In this study, different ratios of α -Fe₂O₃/ZnO/Bentonite NCs were tested against two bacterial strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. The inhibitory zone results showed that the (1:1:1) ratio had the highest antibacterial activity against both bacterial strains compared to other ratios. Therefore, the MIC and MBC values were determined for the (1:1:1) α -Fe₂O₃/ZnO/Bentonite NCs against *E. coli* and *S. aureus*. The MIC of the substance was found to be 0.0781 mg/mL (78.125 μ g/mL) and 0.15625 mg/mL (156.25 μ g/mL) respectively. The MBC is the lowest concentration of the agent that completely inhibits bacterial growth. In other words, it is the minimum amount of an antimicrobial agent needed to eradicate a specific strain of bacteria, which is confirmed by the absence of any colony growth on a growth medium. In our experiment, we observed that the MBC was 0.15625 mg/mL (156.25 μ g/mL) against both *S. aureus* and *E. coli*, indicating that at this concentration, both the MIC and MBC were effective against the bacteria.

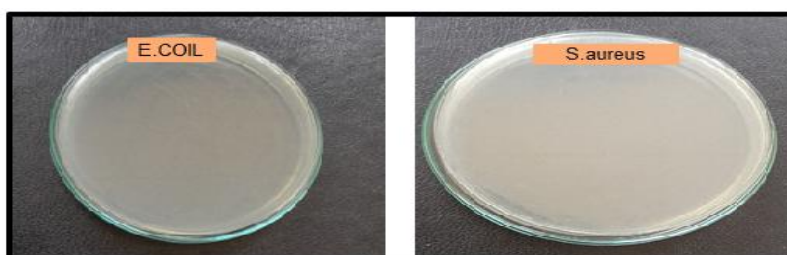


Figure 29. Photograph of MBC result of (1: 1: 1) ZnO/ α -Fe₂O₃//Bentonite.

Table 5: The MIC ($\mu\text{g mL}^{-1}$) and MBC ($\mu\text{g mL}^{-1}$) values were determined for ZnO/ α -Fe₂O₃/Bentonite nanocomposites against the bacterial strains of *E. coli* and *S. aureus*.

Bacterial strain	Strain no	MIC ($\mu\text{g mL}^{-1}$)
<i>E. coli</i>	ATCC 25922	156.25
<i>S. aureus</i>	ATCC 25923	78.125

4.10 Antifungal activity Analysis of the synthesized ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite nanoparticles and nanocomposites

The antifungal activities were investigated for the selected NPs and NCs against *Candida albicans* using the disk diffusion method. The materials were tested in varying concentrations of 10 mg/mL, 5 mg/mL, and 2.5 mg/mL. The results showed that all of the tested materials exhibited antifungal activity against the fungal strains, with inhibition zones ranging from 6-14 mm. The inhibition zones produced by ketoconazole indicated the effectiveness of the antifungal agent against the fungal strains. Negative controls were also included using DMSO solvent to ensure that any observed antifungal activity was not due to the solvent. ZnO NPs showed significant antifungal efficiency against *C. albicans* at a concentration of (1:2), with inhibitory zone diameters of 10 ± 0.37 at 10 mg/mL, 9.5 ± 0.19 at 5mg/mL, and 7 ± 0.21 mm at 2.5mg/mL. These results suggest that the small crystal size of the synthesized ZnO NPs may contribute to their higher antifungal activity and the (1:1) ratio showed the lowest susceptibility, with inhibitory zones of 8.5 ± 0.16 at 10 mg/mL, 7 ± 0.56 mm at 5 mg/mL and 6 ± 0.21 mm at 2.5 mg/mL respectively, as compared to (1:2). The plant extract has shown potential in treating systemic fungal infections, providing a natural alternative to synthetic antifungal medications that often have adverse effects and with inhibitory zones of 19 ± 0.16 at 10 mg/mL, 17 ± 0.56 mm at 5mg/mL and 15 ± 0.21 mm at 2.5mg/mL respectively.

The α -Fe₂O₃ has been identified as having antifungal properties due to its ability to generate reactive oxygen species (ROS) when exposed to light. ROS molecules can damage cells, including those of fungi, and disrupt their normal functions, making hematite a promising candidate for developing new antifungal agents. In this study, α -Fe₂O₃NPs with a ratio of (1:2) were found to have enhanced antifungal properties compared to those with a ratio of (1:1)

against *Candida albicans*. The (1:2) α -Fe₂O₃ NPs showed antifungal activity against *C. albicans*, with inhibition zones of 12 ± 0.81 at 10 mg/mL and 11 ± 0.3 mm at 5 mg/mL, and 10.5 ± 0.3 at 2.5 mg/mL respectively. In contrast, the inhibition zone for (1:1) α -Fe₂O₃ was 10 ± 0.81 at 10 mg/mL and 9 ± 0.3 mm at 5 mg/mL, and 8.5 ± 0.3 at 2.5 mg/mL. These findings suggest hematite NPs are good antifungal activity (Saied et al., 2022). ZnO/bentonite and α -Fe₂O₃/bentonite composites have good antifungal properties.

The (1:1) ZnO/bentonite composite has more antifungal activity than the (1:2) ZnO/bentonite composite. The inhibition zone of (1:1) ZnO/bentonite was 10 ± 0 mm at 10 mg/mL, 9 ± 0.6 at 5 mg/mL, and 8 ± 0.6 mm, while the inhibition zone of (1:2) ZnO/bentonite was 10 ± 0 mm at 10 mg/mL, 9 ± 0.6 at 5 mg/mL, and 8 ± 0.6 mm against *Candida albicans*. Research has shown that the presence of bentonite in zinc oxide enhances the inhibition zone, indicating that ZnO/Bentonite composed from the equal ratio of bentonite and ZnO was more effective for antifungal applications than either bentonite or alone ZnO (de Lucas-Gil et al., 2020). Similarly, the equal ratio of α -Fe₂O₃/bentonite (1:1) has enhanced antifungal activity (de Lucas-Gil et al., 2020). The antifungal property exhibited by the nanocomposite material is attributed to the release of ions such as Fe³⁺, Zn²⁺, Si⁴⁺, Al³⁺, and Mg⁺. In a normal environment, bacterial cell walls are negatively charged due to the presence of carboxyl, hydroxyl, and phosphate groups in the lipid bilayer at the cell surface. The released cations from the nanocomposite bind to these groups, inhibiting normal cellular metabolism and processes. This mechanism is the main reason for the observed antibacterial activity of the nanocomposites. The α -Fe₂O₃/ZnO/Bentonite nanocomposite is particularly effective against *Candida albicans*. As a result, the nanocomposite material can easily penetrate the cell walls and kill the fungi. At the nano-scale, both α -Fe₂O₃ and ZnO NPs possess stronger fungicidal properties than their bulk materials. Their small size allows for better penetration and interaction with fungi, disrupting their cell membrane and increasing their effectiveness as antifungal (Abbas et al., 2020a; Djearamane et al., 2022). When combined with bentonite, these NCs exhibit excellent antifungal activity against *Candida albicans*.

Incorporating bentonite into the composite can reduce the agglomeration characteristics of biosynthesized α -Fe₂O₃ and ZnO NPs, improving the effectiveness of the antifungal treatment. In Figure 34 (1:1:1) ratio of α -Fe₂O₃/ZnO/Bentonite NCs has shown an inhibition zone of 13 ± 0.3 mm at 10 mg/mL, 12 ± 0.3 at 5 mg/mL, and 11 ± 0.3 mm at 2.5 mg/mL against *Candida albicans*. Similarly, the (1:1:2) ratio of α -Fe₂O₃/ZnO/Bentonite NCs has demonstrated an inhibition zone of 10 ± 0 mm at 10 mg/mL, 9 ± 0.3 at 5 mg/mL, and 8 ± 0.3 mm at 2.5 mg/mL against *Candida albicans*. The result demonstrated that the (1:1:1) α -Fe₂O₃/ZnO/Bentonite nanocomposites are more effective in inhibiting fungal growth compared to α -Fe₂O₃, ZnO, and bentonite alone. This is attributed to the high and remarkable antifungal activity exhibited by the (1:1:1) ratio of α -Fe₂O₃ and ZnO against *Candida albicans*, which outperformed other ratios. Furthermore, the equal distribution of α -Fe₂O₃ and ZnO NPs within the structure of bentonite contributed to the enhanced antifungal properties of the NCs.

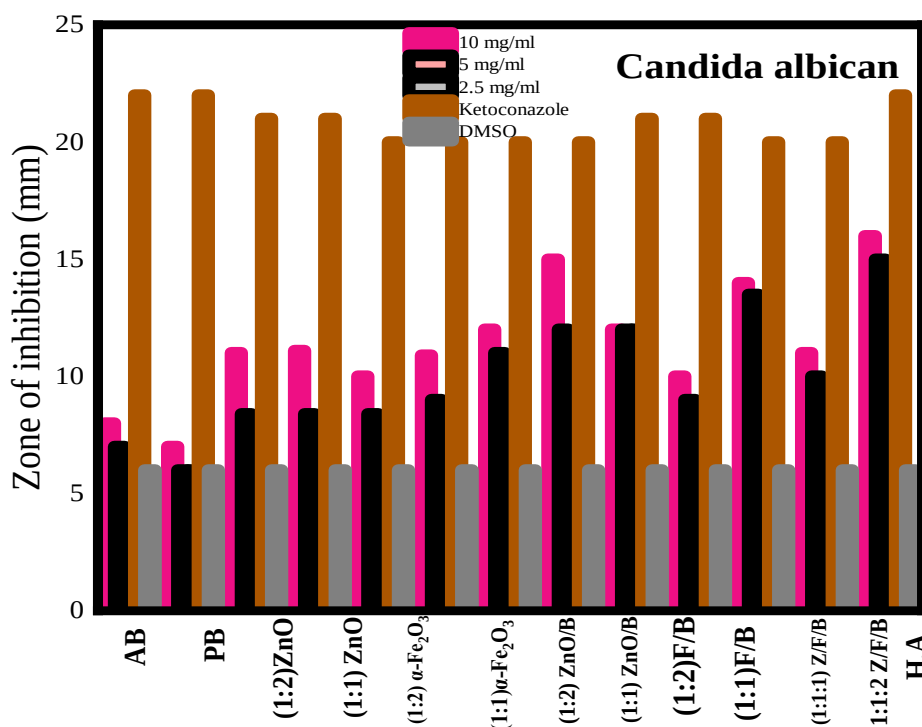


Figure 30. Antifungal activities of purified bentonite, activated bentonite, H. abyssinica ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs, at various ratios, as well as ketoconazole (+ve control) and DMSO (-ve) against *Candida albicans*.

4.10.1 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of (1:1:1) ZnO/ α -Fe₂O₃/Bentonite nanocomposites.

The antifungal activity of α -Fe₂O₃/ZnO/Bentonite NCs was evaluated by determining the Minimum Inhibitory Concentration (MIC) and minimum fungicidal concentration (MFC). The minimum inhibitory concentration (MIC) of the substance against *Candida albicans* was found to be 0.03906mg/mL (39.06 μ g/mL). While the concentration at which no growth of the fungal organism is observed was 0.078 mg/ml and MFCs for *candida albicans* ranged from 0.078 to 10 mg/ml, respectively as indicated in Figure 35. These results provide evidence that the (1:1:1) α -Fe₂O₃/ZnO/Bentonite NCs has strong antifungal activity against *Candida albicans*. Consequently, these findings suggest that NCs is ZnO / α -Fe₂O₃ /Bentonite better antifungal agent for the treatment of fungal infections.

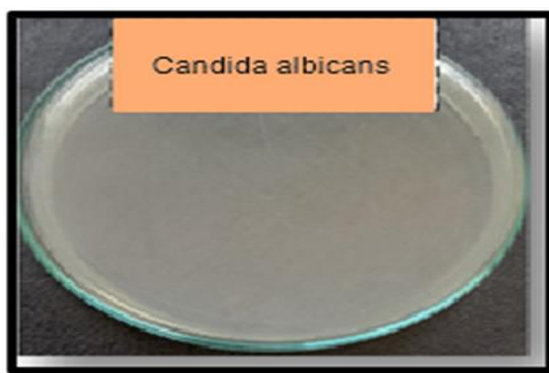


Figure 31. MFC result of (1:1: 1) α -Fe₂O₃/ZnO/Bentonite

Table 6: MIC (μ g/mL⁻¹) and MFC (μ g/mL⁻¹) of (1:1:1) α -Fe₂O₃/ZnO/Bentonite NCs against *Candida albicans*

Bacterial strain	Strain no	MIC (μ g/mL ⁻¹)
<i>Candida albicans</i>	ATCC10231	39.06

CONCLUSION

This work described an eco-friendly, non-toxic, and efficient means of the synthesizing ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and α -Fe₂O₃/ZnO/Bentonite NPs and NCs were developed using an aqueous extract of *H. abyssinica* plant leaf. The synthesized NPs and NCs were characterized by various spectroscopic techniques, revealing their crystalline size, micrographs, and band gap energy, which is crucial for their antimicrobial activity. The biosynthesized ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and α -Fe₂O₃/ZnO/Bentonite were found to exhibit high antibacterial activity against *S. aureus*, *E. coli*, and *Candida albicans*. In general, incorporating ZnO and α -Fe₂O₃ nanoparticles onto the surface of bentonite increased the antibacterial and antifungal activity of the materials. The zone of inhibition observed for the 1:1:1 ratio of α -Fe₂O₃/ZnO/Bentonite NCs against both gram-negative and gram-positive bacteria was greater than that of the other ratios. The synthesis of NPs and NCs using *H. abyssinica* aqueous extracts from natural sources is an eco-friendly and efficient means of producing antimicrobial materials. The study's findings provide insight into the optimization of concentration parameters to enhance the antimicrobial activity of synthesized NPs and NCs, paving the way for future research in the field of the application of metal oxide based nanoparticles as antibacterial and antifungal medicines.

RECOMMENDATION

Based on the findings of the study on ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs and NPs synthesized using *H. abyssinica* plant leaf extract for their antifungal and antibacterial activities, the following recommendations are suggested to benefit researchers in this field:

- The antibacterial and antifungal studies can be extended to include other strains of bacteria and fungi.
- It would be worthwhile Bruneian-Emmett-Teller (BET), to compute the specific surface area of the samples.
- Toxicity testing of the synthesized nanocomposites is necessary to ensure their safety for various applications.
- Win order to gathered additional evidence and establish the characteristics of the nanomaterial, it would be important to conduct such as EDX and TEM of prepared nanomaterials need to be carried out.
- Antioxidant application ZnO, α -Fe₂O₃, ZnO/Bentonite, α -Fe₂O₃/Bentonite, and ZnO/ α -Fe₂O₃/Bentonite NCs and NPs studies could be conducted.
- Photocatalytic activity application of ZnO/ α -Fe₂O₃/Bentonite NCs could also be carried out.

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APPENDIXES

Appendix A

*** Basic Data Process ***

Group	: Standard						
Data	: ZnO-Fe ₂ O ₃ -Bent						
# Strongest 3 peaks							
no. peak	2Theta (deg)	d (Å)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)	Int
1	36.2793	2.47419	100	0.30150	690	11586	
2	31.7925	2.81238	64	0.27990	444	7010	
3	34.4636	2.60027	51	0.31540	352	6227	
# Peak Data List							
peak no.	2Theta (deg)	d (Å)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)	Int
1	15.8798	5.57646	12	0.22550	86	1177	
2	19.8050	4.47922	11	0.65670	74	3030	
3	24.2009	3.67464	7	0.25000	45	1010	
4	26.0362	3.41923	24	0.22600	167	2208	
5	26.7081	3.33510	8	0.29340	52	992	
6	27.5467	3.23544	6	0.40000	38	635	
7	27.8263	3.20357	6	0.37600	39	758	
8	29.4604	3.02948	7	0.19720	50	531	
9	30.6358	2.91588	11	0.21330	75	907	
10	31.7925	2.81238	64	0.27990	444	7010	
11	33.1649	2.69007	20	0.29760	141	2283	
12	34.4636	2.60027	51	0.31540	352	6227	
13	35.0379	2.55966	6	0.23000	39	945	
14	35.6773	2.51455	17	0.33600	117	2632	
15	36.2793	2.47419	100	0.30150	690	11586	
16	37.1360	2.41906	4	0.34000	31	1329	
17	40.5934	2.20664	3	0.12000	21	189	
18	40.9782	2.20582	6	0.21000	40	487	
19	47.5686	1.91002	19	0.32650	132	2489	
20	47.9892	1.89426	3	0.13340	23	323	

Figure 1: Information for XRD analysis of ZnO/ α -Fe₂O₃/ Bentonite.

Appendix B

Table 1. Results of Interlayer Spacing and Composition of α -Fe₂O₃.

Sample	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:2α-Fe₂O₃	33.1	2.7	0.409	0.999	19.395	20.2	(104)
	35.7	2.5	0.334	0.9999	23.740		(110)
	54.0	1.7	0.448	0.9999	17.741		(116)
1:1 α-Fe₂O₃	33.2	2.7	0.258	0.999	30.700	28.7	(104)
	35.7	2.5	0.270	0.999	29.383		(110)
	54.1	1.7	0.304	0.999	26.085		(116)

Table 2. Results of Interlayer Spacing and Composition of ZnO NPs

Sample	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:2 ZnO	36.2	2.4	0.3605	0.999987	22.05	22.7	(100)
	31.7	2.8	0.3379	0.999990	23.53		(002)
	34.4	2.6	0.3513	0.999988	22.63		(101)

2:1 ZnO	36.3	2.5	0.3059	0.999986	25.98	27.3	(100)
	31.8	2.8	0.2784	0.999988	28.54		(002)
	34.5	2.6	0.2893	0.999987	28.54		(101)
1:1 ZnO	36.3	2.5	0.3072	0.999986	25.873	26.1	(100)
	31.8	2.8	0.2930	0.999987	27.127		(002)
	34.5	2.6	0.3122	0.99998	25.459		(101)

Table 3. Results of Interlayer Spacing and Composition ZnO/Bent.

Sample	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:1 ZnO/Bent	36.3	2.5	0.6750	0.999931	11.77527	12.2	(100)
	34.5	2.6	0.6636	0.999933	11.97755		(002)
	31.8	2.8	0.6175	0.999942	12.87171		(101)
2:1 ZnO/Bent	36.3	2.5	0.3072	0.999986	25.87343	26.1	(100)
	31.8	2.8	0.2930	0.999987	27.12737		(002)
	34.5	2.6	0.3122	0.999985	25.45906		(101)
1:2 ZnO/Bento	36.3	2.5	0.3059	0.999986	25.98338	27.3	(100)
	31.8	2.8	0.2784	0.99998	28.5495		(002)
	34.5	2.6	0.2893	0.999987	28.5495		(101)

Table 4. Results of Interlayer Spacing and Composition α -Fe₂O₃/Bent NCs

Sample	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:1 α -Fe ₂ O ₃ /Bent	33.1	2.7	0.4098	0.999974	19.39560	20.2	(104)
	35.7	2.5	0.3348	0.999983	23.74049		(110)
	54.0	1.7	0.4480	0.999969	17.74178		(116)
2:1 α -Fe ₂ O ₃ /Bent	33.1	2.7	0.4098	0.999974	19.39560	20.4	(104)
	35.7	2.5	0.3348	0.999983	23.74049		(110)
	54.0	1.7	0.4480	0.999969	17.74178		(116)

1:2	α -	33.2	2.7	0.2829	0.999988	28.09585	30.1	(120)
Fe ₂ O ₃ /Bent		35.7	2.5	0.2900	0.999987	27.40799		(104)
		26.1	3.4	0.2267	0.999992	35.06095		(110)

Table 5. Results of Interlayer Spacing and Compositions ZnO/ α -Fe₂O₃/Bent NCs

Samples	Strong Peak position (2 θ (°))	d(Å)	FWHM (°)	β (rad)	D(nm)	Average D (nm)	(hkl)
1:1:1	34.7	2.6	1.0320	0.99983	7.701859	10.36	(100)
ZnO/ α -	26.1	3.4	0.6467	0.99993	12.29058		(104)
Fe ₂ O ₃ /Bent	19.8	4.8	0.7150	0.99992	11.11652		(114)
2:2:1	34.7	2.6	0.6086	0.999944	13.060004	16.04	(111)
ZnO/ α -	26.7	3.3	0.5371	0.999956	14.798582		(101)
Fe ₂ O ₃ /Bent	21.1	4.2	0.3919	0.999977	20.281496		(221)
1:1:3	36.3	2.5	0.3015	0.999986	26.362582	28.87	(100)
ZnO/ α -	26.1	3.4	0.2267	0.999992	35.060955		(110)
Fe ₂ O ₃ /Bent	34.5	2.6	0.3154	0.999985	25.200756		(101)

Appendix C

Antibacterial activity of *H. abyssinica*, bentonite, ZnO, α -Fe₂O₃, ZnO/Bent α -Fe₂O₃/Bent ,ZnO/ α -Fe₂O₃/Bent NCs

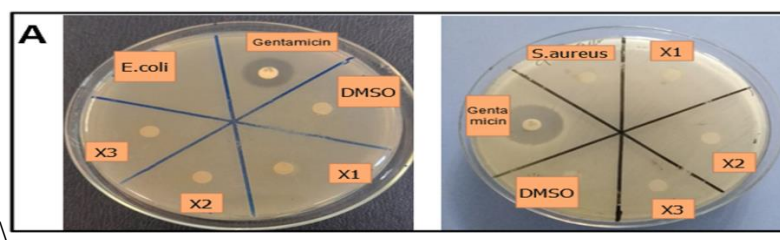


Figure 2. Photographs of antibacterial activities of a (PB) and b (Na-AB).

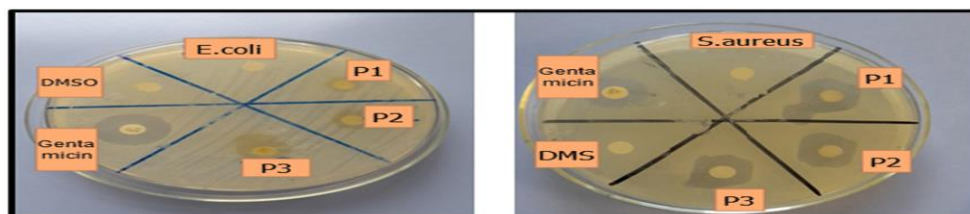


Figure 3. Photographs of antibacterial activities of P (*H. abyssinica* Plant leaf extract).

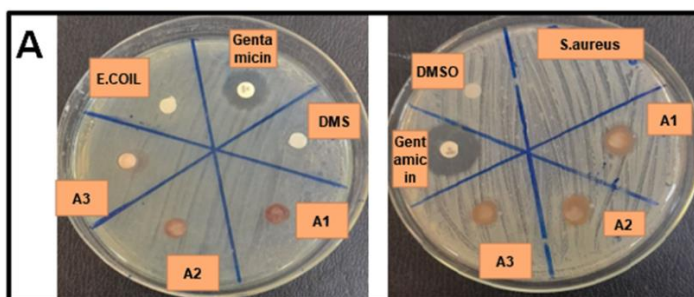


Figure 3. Photographs of antibacterial activities of A1 (1:2 α -Fe₂O₃ NPs).

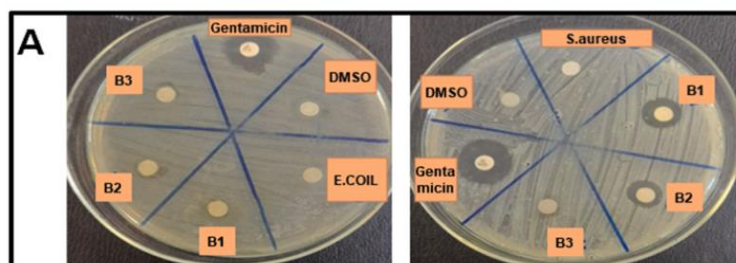


Figure 4. Photographs of antibacterial activities of B (1:2) (ZnO NPs) respectively.

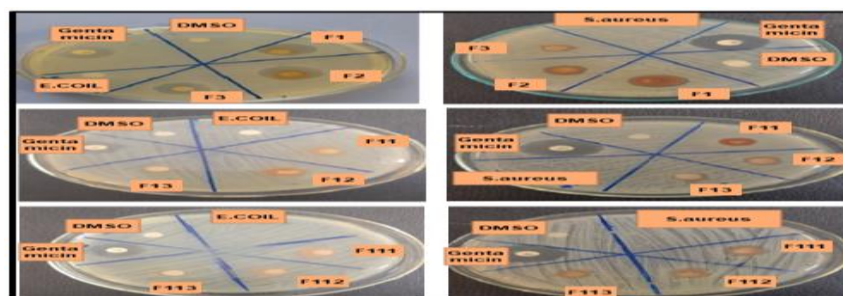


Figure5. Photographs of antibacterial activities of F1 (1:1), F11 (1:2) and F111 (2:1) of α -Fe₂O₃ /Bentonite.

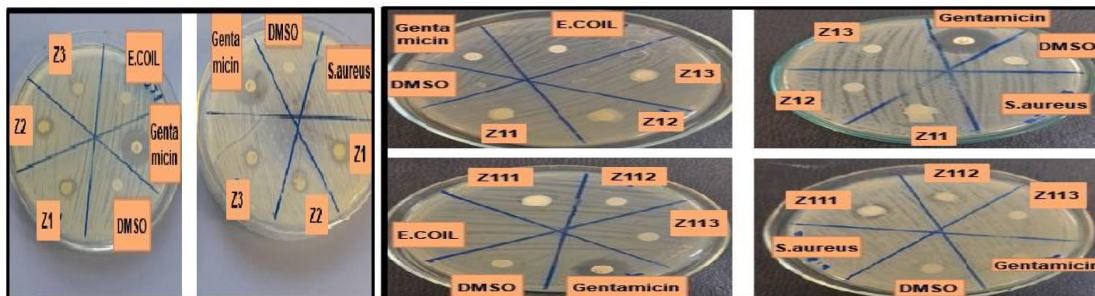


Figure 6. Photographs of antibacterial activities of Z1 (1:1), Z11 (2:1) and Z111 (1:2) ZnO/bentonite.

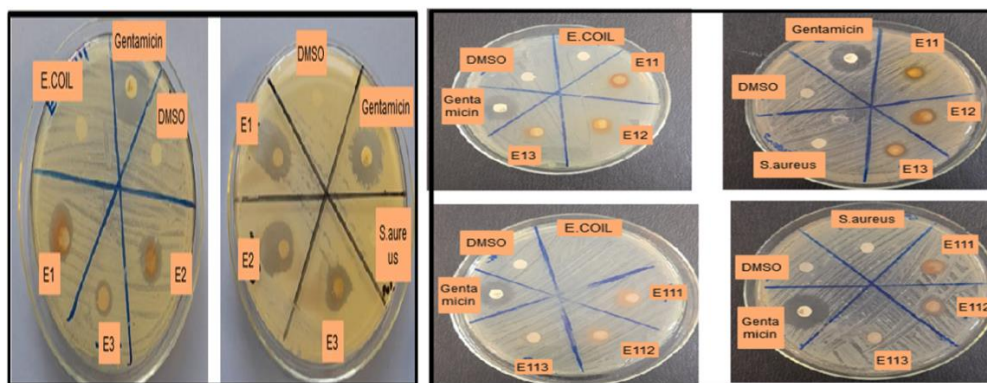


Figure 7. Photographs of antibacterial activities of E1 (1:1:1), E11 (2:2:1) and E111 (1:1:3) of α -Fe₂O₃/ZnO/Bentonite.

Appendix C

Antifungal activity of *H. abyssinica*, bentonite, ZnO, α -Fe₂O₃, ZnO/Bent α -Fe₂O₃/Bent, ZnO/ α -Fe₂O₃/Bent NCs

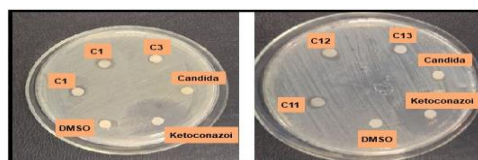
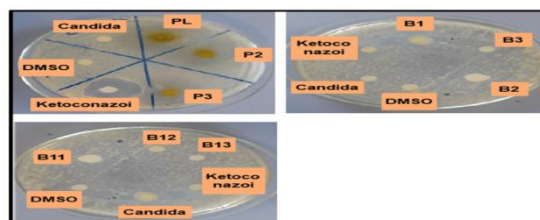


Figure 8. Photographs of antifungal activities of C1 (1:1) and C (1:2) of ZnO NPs.



Types	Dilution Results, Means of three replications and Standard error ($\pm$ SE) of Inhibition zones in mm						
No	Types of NPs/N Cs	Types of Organism	10mg/mL (A)	5mg/mL (B)	2.5mg/mL (C)	Gentamicin (+)	DMSO (-ve)
1	Activated bentonite	<i>S. aureus</i> ATCC25923	6 $\pm$ 0.3	6 $\pm$ 0.3	6 $\pm$ 0.3	24 $\pm$ 0	6 $\pm$ 0
		<i>E. coli</i> ATCC25923	6 $\pm$ 0.6	6 $\pm$ 0.3	6 $\pm$ 0.3	6 $\pm$ 0.3	6 $\pm$ 0
	Purified bentonite	<i>E. coli</i> ATCC25923	6 $\pm$ 0.1	6 $\pm$ 0.3	6 $\pm$ 0.3	6 $\pm$ 0.3	6 $\pm$ 0
2	Fe ₂ O ₃ (1:1) (1:2)	<i>S. aureus</i> ATCC25923	10 $\pm$ 0.3 mm,	9 $\pm$ 0.3 mm	8 $\pm$ 0.3	16 $\pm$ 0.3	6 $\pm$ 0
		<i>E. coli</i>	8 $\pm$ 0.3 mm,	7 $\pm$ 0.3 ,	6 $\pm$ 0.3 mm	17 $\pm$ 0.3	6 $\pm$ 0
		<i>E. coli</i> <i>S. aureus</i>	11 $\pm$ 0.3 mm,	10 $\pm$ 0.3 mm,	8 $\pm$ 0.3 mm	25 $\pm$ 0.6	6 $\pm$ 0
			9 $\pm$ 0.3 mm,	8 $\pm$ 0.3 mm,	7 $\pm$ 0.3 mm	25 $\pm$ 0.6	6 $\pm$ 0
3	ZnO (1:1) (1:2) (2:1)	<i>S. aureus</i>	12 $\pm$ 0.3 mm	10 $\pm$ 0.3 mm	7 $\pm$ 0.3 mm		
		<i>E. coli</i>	9 $\pm$ 0.3	8 $\pm$ 0.3	7 $\pm$ 0.3		
		<i>S. aureus</i>	13 $\pm$ 0.3	12 $\pm$ 0.3	11 $\pm$ 0.3		
		<i>E. coli</i>	10 $\pm$ 0.3	9 $\pm$ 0.3	8 $\pm$ 0.3		
3	(1:1) ZnO /Bentonite (1:2) (2:1)	<i>S. aureus</i>	14 $\pm$ 0.3	10 $\pm$ 0.6	9 $\pm$ 0.6	17 $\pm$ 0.3	6 $\pm$ 0
		<i>E. coli</i>	12 $\pm$ 0.3	10 $\pm$ 0.6	7 $\pm$ 0.6	17 $\pm$ 0.3	6 $\pm$ 0
		<i>S. aureus</i>	8.5 $\pm$ 0.3	7.5 $\pm$ 0.6	6 $\pm$ 0.6	15 $\pm$ 0.6	6 $\pm$ 0
		<i>E. coli</i>	10 $\pm$ 0.3	9.5 $\pm$ 0.3	8 $\pm$ 0.6	15 $\pm$ 0.6	6 $\pm$ 0

		S. aureus	10±0.3	8±0.6	7.5±0.6	22±0.3	6±0
		E. coli	9±0.3	8±0.6	7± 0.6	22±0.3	6±0
4	(1:1) α-Fe₂O₃/Bent	S. aureus	15 ± 0.6	10±0.3	8± 0.3	22±0.6	6±0
		E. coli	14 ±0.3	13.3±0.6	12± 0.6	22±0.6	6±0
	(1:2) (2:1)	S. aureus	10± 0.6	9 ±0.3	8±0.3	17±0.3	6±0
		E. coli	10±0.3	9± 0.6	8 ± 0.6	17±0.3	6±0
	(1:1:1) Z/F/B	S. aureus	17±0.3	16±0.6	14±0.6	25±0.6	6±0
		E. coli	14±0.3	13±0.6	12±0.6	25±0.6	6±0
	1:1:2	S. aureus	13±0.6	12±0.6	11±0.6	25±0.6	6±0
		E. coli	10±0.6	9.5±0.3	8±0.6	25±0.6	6±0
4	2:2:1	S. aureus	10±0.6	8±0.6	7.5±0.6	24±0.6	6±0
		E. coli	9±0.6	8±0.6	7.5±0.6	24±0.6	6±0
	H.A Plant leaf extract	S. aureus	19±0.3	17±0.3	18±0.3	22±0.3	6±0
		E. coli	16±0.3	15±0.3	14±0.6	22±0.3	6±0

Appendix D

Inhibition zone of Antifungal activity of *H. abyssinica*, bentonite, ZnO, α-Fe₂O₃, ZnO/Bent α-Fe₂O₃/Bent, ZnO/ α-Fe₂O₃/Bent NCs

N Dilution Results, Means of three replications and Standard error (±SE) of Inhibition zones							
o in mm							
	Types of NPs/NCs	Types	10mg/m L (A)	5mg/mL (B)	2.5mg/mL (C)	Ketoconazole (+ve)	DMSO (-ve)
1	AB	Candida albicans	8±0.3	7±0.6	6±0.28	22±0	6
	PB		7±0.6	6±0.6	6±0.6	22±0	6
2	Zno (1:2)		11.1±05	8.4±0.6	6.6±0.25	21±0.3	6
	(1:1)		11.1±05	8.4±0.6	6.4±0.76	21±0.3	6
	Fe ₂ O ₃ (1:2)		10.9±0.5	9.13±0.7	7.4 ±0.81	20±0.3	6
	(1:1)		10.±0.5	8.4±0.7	7.13± 0.81	20±0.3	6
3	ZnO/bent (1:2		12±0.6	11±0.6	10±0.6	20±0.3	6
	1;1		15±0.6	12±0.6	11±0.6	20±0.3	6
	Fe ₂ O ₃ /bent (1:1)		12±0.6	12±0.6	12±0.6	21±0.3	6
	1:2		10±0.6	9±0.6	8±0.6	21±0.3	6
	Zno/Fe ₂ O ₃ /bent		14±0.5	13.5±0.2	11±0.3	20±0.3	6
	(1:1:1)						
	(1:1:2)		11±0.5	10±0.5	9±0.5	20±0.3	6
4	H. Abyssinica Plant		16±0.11	15±-.3	14±0.3	22±0.3	6

