

**Sudan University of Science and Technology**

**College of Graduate Studies**

**Synthesis and Characterization of Some Antibiotic-Silver  
Nanoparticles Complexes and their Antibacterial Efficiency**

**تحضير وتوصيف معقدات الفضة النانوية مع بعض المضادات الحيوية ودراسة  
كفاءتها تجاه البكتريا**

A Thesis Submitted in Fulfillment of the Requirements for the Degree

Of Doctor of Philosophy in Chemistry

By

**Bedour Badr Musa**

B.Sc, H.D and M.Sc. (Chemistry)

Supervisor: Dr. Mohammed Adam Abbo

Co- Supervisor: Dr. Essa Esmail

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## Chapter One

### 1.1 Introduction

Over the last few decades, the applications of nanotechnology in medicine have been extensively explored in many medical areas, especially in drug delivery. Nanotechnology concerns the understanding and control of matters in the 1-100 nm range, at which scale materials have unique physicochemical properties including ultra-small size, large surface to mass ratio, high reactivity and unique interactions with biological systems [1]. Drug-loaded nanoparticles can be achieved through physical encapsulation, adsorption, or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts. Many advantages of nanoparticle-based drug delivery have been recognized, including improving serum solubility of the drugs, prolonging the systemic circulation lifetime, releasing drugs at a sustained and controlled manner, preferentially delivering drugs to the tissues and cells of interest, and concurrently delivering multiple therapeutic agents to the same cells for combination therapy [2]. Moreover, drug-loaded nanoparticles can enter host cells through endocytosis and then release drug payloads to treat microbes-induced intracellular infections. As a result, a number of nanoparticle-based drug delivery systems have been approved for clinical uses to treat a variety of diseases and many other therapeutic nanoparticle formulations are currently under various stages of clinical tests [3].

Using heavy metals in nanoparticle forms to counter bacteria drug resistance is one of the strategies that have been intensively investigated. Silver has been used as an antibacterial agent for more than 2000 years. It

is still used today to control microorganism growth in a variety of applications including dental, catheters, clothing, and wound healing. It is well-known that silver ions and silver nanoparticles (Ag NPs) are toxic to microorganisms, including at least 16 major species of bacteria [4]. Ag NPs are reported to possess antifungal, antiviral, antigenic, and anti-inflammatory properties, and have a synergistic effect against bacteria when combined with some antibiotics [5]. It has been shown that the combination of amoxicillin and Ag NPs has greater bactericidal activity against *E. coli* than when applied separately. Interactions between Ag NPs and polymyxin B showed synergistic effects for gram-negative *E. coli* and *S. aureus*. Ag NPs produced biosynthetically have been shown to have good antibiotic activities, although the combination of antibiotics with Ag NPs has been shown to increase some antibiotics' efficacy against pathogens, its influence on the multidrug-resistant *Salmonella typhimurium* DT104 is unknown [6]. Studies the antibacterial activity of Ag NPs in combination with three classes of antibiotics—polykeptide (tetracycline),  $\beta$ -lactam (penicillin), and aminoglycoside (neomycin) against *Salmonella typhimurium* DT104 were done [7].

This upsurge in exploring alternative approaches to infection treatment has also raised the possibility of using Ag NPs with improved pharmacokinetics and even pharmacodynamics

Silver ions and silver-based compounds are highly toxic to microorganisms. Thus, silver ions have been used in many kinds of formulations [8] and recently it was shown that hybrids of AgNPs with amphiphilic hyperbranched macromolecules exhibit effective antimicrobial surface coating [9]. The most important application of silver and AgNPs is in the medical industry, such as topical ointments to prevent infection in burns and open wounds [10]. Newly devised AgNPs-coated wound

dressings have been a major breakthrough in the management of wounds or infections. To prevent or reduce infections, a new generation of dressings incorporating antimicrobial agents like silver has been developed [11]. Impregnation of wound dressings with colloidal silver resulted in a strong decrease of pathogen specific alterations in infected epithelium. The delivery of silver and AgNPs to infected keratinocytes in a moist healing environment is efficient, fast, and active as compared to wound dressing without silver [12]. Similar results with *E. coli* were obtained with AgNPs.

In recent years, encapsulation of antimicrobial drugs in nanoparticle systems has emerged as an innovative and promising alternative that enhances therapeutic effectiveness and minimizes undesirable side effects of the drugs.

In addition, nanosilver has excellent properties of conformational entropy in polyvalent binding, which make it easy to attach to flexible polymeric chains of antibiotics. Moreover, nanosilver possesses well developed surface chemistry, chemical stability, and appropriate size (20 nm in diameter, 250 times smaller than a bacterium). It is able to maintain constant shape and size in solution. Thus it is a good choice to use nanosilver as inorganic nanomaterials in combination with  $\beta$ -lactam, as the inorganic nanomaterials against *E. coli* cells.

Antibiotic resistance is one of the biggest threats to modern society. The medicines used to treat dangerous bacterial infections are rapidly losing effectiveness that creating side effects. Nowadays, scientists have found that bacteria are less prone to develop resistance against silver nanoparticles (Ag NPs) than conventional antibiotics [13].

## **1.2. Objectives and scope of this study**

The objectives of this study are:

- To prepare antibiotic-Ag nanoparticles complexes.
- To characterize the complexes prepared.
- To modify and organize the Ag nanoparticles through complexation with some pharmaceutical drugs.
- To introduce new biofunctional silver complexes to be used as an antibacterial active compound.

## CHAPTER TWO

### Literature Review

#### 2.1 Nanotechnology

Nanotechnology literally means any technology on a nano-scale that has applications in the real world. Nanotechnology encompasses the production and application of physical, chemical, and biological systems at scales ranging from individual atoms or molecules to submicron dimensions, as well as the integration of the resulting nanostructures into larger systems. [14]

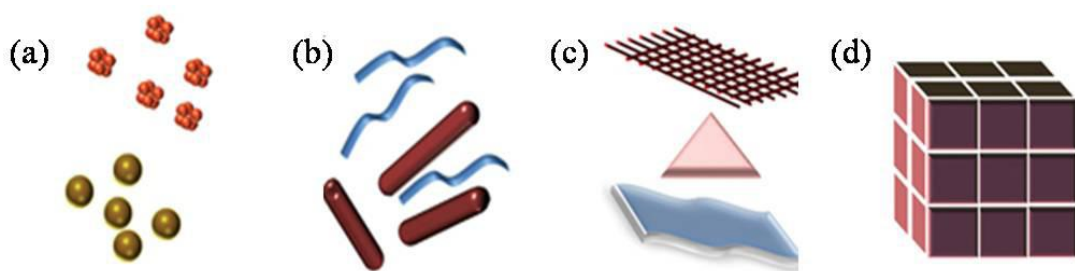
Silver nanoparticles are of interest because of the unique properties (*e.g.*, size and shape depending optical, electrical, and magnetic properties) which can be incorporated into antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components [15]

Several physical and chemical methods have been used for synthesizing and stabilizing silver nanoparticles. The most popular chemical approaches, including chemical reduction using a variety of organic and inorganic reducing agents, electrochemical techniques, physicochemical reduction, and radiolysis are widely used for the synthesis of silver nanoparticles [16]. Recently, nanoparticle synthesis is among the most interesting scientific areas of inquiry, and there is growing attention to produce nanoparticles using environmentally friendly methods (green chemistry) [17].

### 2.1.1 Classification of Nanomaterials

Nanomaterials have extremely small size which having at least one dimension 100 nm or less. Nanomaterials can be nanoscale in one dimension (e.g. surface films), two dimensions (e.g. strands or fibers), or three dimensions (e.g. particles). They can exist in single, fused, aggregated or agglomerated forms with spherical, tubular, and irregular shapes. Common types of nanomaterials include nanotubes, dendrimers, quantum dots and fullerenes. Nanomaterials have applications in the field of nanotechnology, and displays different physical chemical characteristics from normal chemicals (i.e., silver nanoparticles, carbon nanotube, fullerene, photocatalyst, carbon nanoparticles, and silica).

According to Siegel [18], Nanostructured materials are classified as Zero dimensional, one dimensional, two dimensional, three dimensional nanostructures [19].



**Fig.2.1 Classification of Nanomaterials (a) 0-D spheres and clusters, (b) 1-D nanofibers, wires, and rods, (c) 2-D films, plates, and networks, (d) 3-D nanomaterials.**

Nanomaterials are materials which are characterized by an ultra fine grain size ( $< 50$  nm) or by a dimensionality limited to 50 nm. Nanomaterials can

be created with various modulation dimensionalities as defined by Richard W. Siegel [19]: zero dimensional (atomic clusters, filaments and cluster assemblies), one dimensional (multilayer), two dimensional (ultrafine-grained over layers or buried layers) and three dimensional (nanophase materials consisting of equiaxed nanometer sized grains) as shown in the above Figure [1].

### **2.1.2 Properties of nanomaterials**

Nanomaterial possesses superior physical properties due to their ultrahigh surface, ultrahigh volume, and quantum size effect along with their magnetic properties. Nanoscale materials have far larger surface areas than similar masses of larger-scale materials. As surface area per mass of a material increases, a greater amount of the material can come into contact with surrounding materials, thus affecting reactivity [20]. When particles are synthesized with dimensions of about 1–100 nanometers, the materials' properties change significantly, from those of the bulk. This is the size scale where so-called quantum effects rule the behavior and properties of particles. Properties of materials are size-dependent in this scale range [21].

Thus, when particle size is reduced to nanoscale, properties such as melting point, fluorescence, electrical conductivity, magnetic permeability, and chemical reactivity change as a function of the size of the particle. A fascinating and powerful result of the quantum effects of the nanoscale is the concept of “tunability” of properties. That is, by changing the size of the particle, a scientist can, literally, fine-tune a material property of interest (e.g., changing fluorescence color) [22].

### **2.1.3 Applications of Nanotechnology**

The different fields that find potential applications of nanotechnology are as follows:



- a. Health and Medicine
- b. Electronics
- c. Transportation
- d. Energy and Environment
- e. Space exploration

### **2.1.4 Background of Ag nanoparticle**

Over the last decades silver has been engineered into nanoparticles, structures from 1 to 100 nm in size. Owing to their small size, the total surface area of the nanoparticles is maximized, leading to the highest values of the activity to weight ratio [23]. Due to this property being distinctly different from that of the bulk metal, silver nanoparticles have attracted much attention and have found applications in antibacterial/antifungal agents in a diverse range of consumer products: air sanitizer sprays, socks, pillows, slippers, respirators, wet wipes, detergents, soaps, shampoos, toothpastes, air filters, coatings of refrigerators, vacuum cleaners, washing machines, food storage containers, cellular phones [24].

Numerous synthesis approaches were developed to obtain silver nanoparticles electron irradiation [25], chemical reduction by inorganic and organic reducing compared minimum inhibitory concentration (MIC) values for bacterial cultures, one can see that the antimicrobial activity of silver nanoparticles strongly depends on the method of their synthesis [26].

## **2.2. Synthesis of silver nanoparticles**

### **2.2.1 Physical approaches**

Most important physical approaches include evaporation-condensation and laser ablation [27]. Various metal nanoparticles such as silver, gold, lead sulfide, cadmium sulfide, and fullerene have previously been synthesized using the evaporation-condensation method.

The absence of solvent contamination in the prepared thin films and the uniformity of nanoparticles distribution are the advantages of physical approaches in comparison with chemical processes. Physical synthesis of silver nanoparticles using a tube furnace at atmospheric pressure has some disadvantages [28], for example, tube furnace occupies a large space, consumes a great amount of energy while raising the environmental temperature around the source material, and requires a great deal of time to achieve thermal stability. Moreover, a typical tube furnace requires power consumption of more than several kilowatts and a long preheating time to reach a stable operating temperature. It was demonstrated that silver nanoparticles could be synthesized via a small ceramic heater with a local heating source [29]. The evaporated vapor can cool at a suitable rapid rate, because the temperature gradient in the vicinity of the heater surface is very steep in comparison with that of a tube furnace. This makes possible the formation of small nanoparticles in high concentration. This physical method can be useful as a nanoparticle generator for long-term experiments for inhalation toxicity studies, and as a calibration device for nanoparticle measurement equipment [29].

Silver nanoparticles could be synthesized by laser ablation of metallic bulk materials in solution. The ablation efficiency and the characteristics of produced silver nanoparticles depend upon many factors such as the wavelength of the laser impinging the metallic target, the duration of the laser pulses (in the femto-, pico- and nanosecond regime), the laser fluence, the ablation time duration and the effective liquid medium, with or without the presence of surfactants [30]. One important advantage of laser ablation technique compared to other methods for production of metal colloids is the absence of chemical reagents in solutions. Therefore, pure and

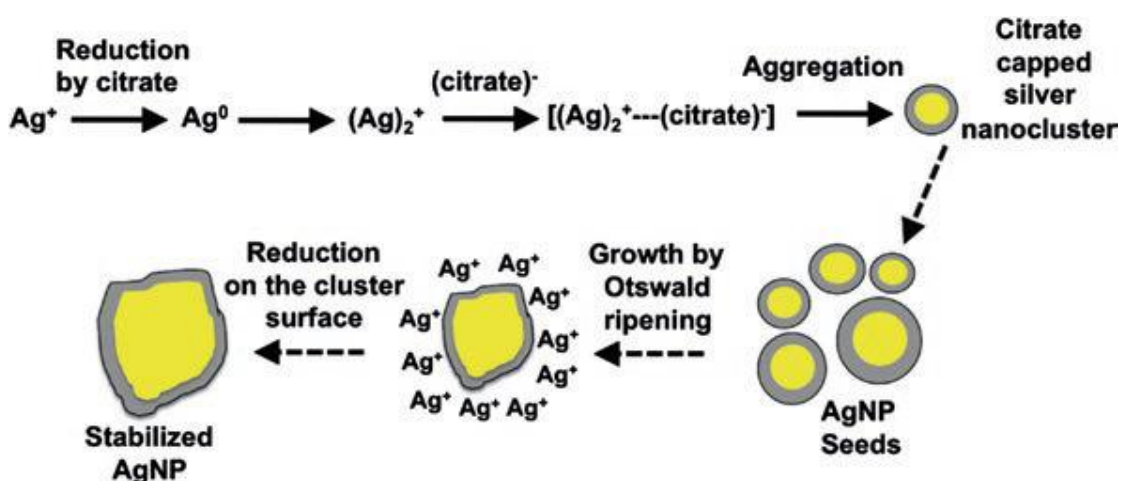
uncontaminated metal colloids for further applications can be prepared by this technique [31].

Silver nanospheroids (20 – 50 nm) were prepared by laser ablation in water with femtosecond laser pulses at 800 nm [32]. The formation efficiency and the size of colloidal particles were compared with those of colloidal particles prepared by nanosecond laser pulses. The results revealed the formation efficiency for femtosecond pulses was significantly lower than that for nanosecond pulses. The size of colloids prepared by femtosecond pulses were less dispersed than that of colloids prepared by nanosecond pulses. Furthermore, it was found that the ablation efficiency for femtosecond ablation in water was lower than that in air, while, in the case of nanosecond pulses, the ablation efficiency was similar in both water and air.

### 2.2.2 Chemical approaches

The most common approach for synthesis of silver nanoparticles is chemical reduction by organic and inorganic reducing agents. In general, different reducing agents such as sodium citrate, ascorbate, sodium borohydride ( $\text{NaBH}_4$ ), elemental hydrogen, polyol process, Tollens reagent, N, N-dimethylformamide (DMF), and poly (ethylene glycol)-block copolymers are used for reduction of silver ions ( $\text{Ag}^+$ ) in aqueous or non-aqueous solutions Fig 2.1.

The aforementioned reducing agents reduce silver ions ( $\text{Ag}^+$ ) and lead to the formation of metallic silver ( $\text{Ag}^0$ ), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to formation of metallic colloidal silver particles [33].



**Fig.2.2 Nucleation and growth mechanisms for AgNPs [34]**

It is important to use protective agents to stabilize dispersive nanoparticles during the course of metal nanoparticle preparation, and protect the nanoparticles that can be absorbed on or bind onto nanoparticle surfaces, avoiding their agglomeration [35]. Polymeric compounds such as poly(vinylalcohol), poly(vinylpyrrolidone), poly(ethyleneglycol), poly(methacrylic acid), and poly(methylmethacrylate) have been reported to be effective protective agents to stabilize nanoparticles.

In one study, [36], dodecanethiol capped silver nanoparticles were prepared according to Brust [37], based on a phase transfer of an  $\text{Au}^{3+}$  complex from aqueous to organic phase in a two-phase liquid liquid system, followed by a reduction with sodium borohydride in the presence of dodecanethiol as a stabilizing agent, binding onto the nanoparticles surfaces, thereby avoiding their aggregation and making them soluble in certain solvents. It was reported [37] that small changes in synthetic factors lead to dramatic modifications in nanoparticle structure, average size, size distribution width, stability and self-assembly patterns.

Uniform and size controllable silver nanoparticles can be synthesized using micro-emulsion techniques. The nanoparticles preparation in two-phase aqueous organic systems is based on the initial spatial separation of reactants (metal precursor and reducing agent) in two immiscible phases. The interface between the two liquids and the intensity of inter-phase transport between two phases, which is mediated by a quaternary alkyl-ammonium salt, affect the rate of interactions between metal precursors and reducing agents.

Metal clusters formed at the interface are stabilized, due to their surface being coated with stabilizer molecules occurring in the non-polar aqueous medium, and transferred to the organic medium by the inter-phase transporter [38]. One of the major disadvantages of this method is the use of highly deleterious organic solvents. Thus large amounts of surfactant and organic solvent must be separated and removed from the final product.

A simple and effective method, UV-initiated photoreduction, has been reported for synthesis of silver nanoparticles in the presence of citrate, polyvinylpyrrolidone, poly(acrylic acid), and collagen. For instance, Huang and Yang [39] produced silver nanoparticles via the photoreduction of silver nitrate in layered inorganic laponite clay suspensions which served as a stabilizing agent for the prevention of nanoparticles aggregation. The properties of the produced nanoparticles were studied as a function of UV irradiation time. Silver nanoparticles (nanosphere, nanowire, and dendrite) have been prepared by an ultraviolet irradiation photoreduction technique at room temperature using poly(vinylalcohol) (as protecting and stabilizing agent). Concentration of both poly(vinylalcohol) and silver nitrate played significant role in the growth of the nanorods and dendrites [40].

Nano-sized silver particles with an average size of 8 nm were prepared by photoinduced reduction using poly(styrene sulfonate)/poly(allylamine hydrochloride) polyelectrolyte capsules as microreactors [41]. Moreover, it was demonstrated that the photoinduced method could be used for converting silver nanospheres into triangular silver nanocrystals (nanoprisms) with desired edge lengths in the range of 30–120 nm [42]. The particle growth process was controlled using dual-beam illumination of nanoparticles.

Electrochemical synthetic method can be used to synthesize silver nanoparticles. It is possible to control particle size by adjusting the electrolysis parameters and to improve homogeneity of silver nanoparticles by changing the composition of the electrolytic solutions. Polyphenylpyrrole-coated silver nanospheroids (3–20 nm) were synthesized by electrochemical reduction at the liquid/liquid interface. This nano-compound was prepared by transferring the silver metal ion from the aqueous phase to the organic phase, where it reacted with pyrrole monomer. [43]

Silver nanoparticles can be synthesized by using a variety of irradiation methods. Laser irradiation of an aqueous solution of silver salt and surfactant can produce silver nanoparticles with a well-defined shape and size distribution [44].

Microwave assisted synthesis is a promising method for the synthesis of silver nanoparticles. It was reported that silver nanoparticles could be synthesized by a microwave-assisted synthesis method employing carboxymethyl cellulose sodium as a reducing and stabilizing agent.

The size of the resulting particles depended on the concentration of sodium carboxymethyl cellulose and silver nitrate. The produced nanoparticles

were uniform and stable, and were stable at room temperature for 2 months without any visible change. [45]

Green synthesis of silver nanoparticles using negatively charged heparin (reducing/stabilizing agent and nucleation controller) was also reported by heating a solution of silver nitrate and heparin to 70 °C for approximately 8 h [46]. Transmission electron microscopy (TEM) micrographs demonstrated an increase in particle size of silver nanoparticles with increased concentrations of silver nitrate (as the substrate) and heparin. Moreover, changes in the heparin concentration influenced the morphology and size of silver nanoparticles. The synthesized silver nanoparticles were highly stable, and showed no signs of aggregation after two months [46]

Recently, a simple one-step process, Tollens method, has been used for the synthesis of silver nanoparticles with a controlled size. This green synthesis technique involves the reduction of  $\text{Ag}(\text{NH}_3)_2^+$  (as a Tollens reagent) by an aldehyde [37]. In the modified Tollens procedure, silver ions are reduced by saccharides in the presence of ammonia, yielding silver nanoparticle films (50-200 nm), silver hydrosols (20-50 nm) and silver nanoparticles of different shapes. In this method, the concentration of ammonia and the nature of the reducing agent play an important role in controlling size and morphology of the silver nanoparticles. It was revealed that the smallest particles were formed at the lowest ammonia concentration. Glucose and the lowest ammonia concentration (5 mM) resulted in the smallest average particle size of 57 nm with an intense maximum surface plasmon absorbance at 420 nm. Moreover, an increase in  $\text{NH}_3$  from 0.005 M to 0.2 M resulted in a simultaneous increase in particle size and polydispersity [48]. Silver nanoparticles with controllable sizes were synthesized by reduction of  $[\text{Ag}(\text{NH}_3)_2]^+$  with glucose, galactose, maltose, and lactose [49]. The nanoparticle synthesis was carried out at various ammonia

concentrations (0.005-0.20 M) and pH conditions (11.5-13.0), resulting in average particle sizes of 25-450 nm. The particle size was increased by increasing (NH<sub>3</sub>), and the difference in the structure of the reducing agent (monosaccharides and disaccharides) and pH (particles obtained at pH 11.5 were smaller than those at pH 12.5) influenced the particle size. Polydispersity also decreased in response to decreases the pH. Produced silver nanoparticles were stabilized and protected by sodium dodecyl sulfate (SDS), polyoxyethylenesorbitane monooleate, and polyvinylpyrrolidone (PVP) [50].

### **2.2.3 Biological approaches**

In recent years, the development of efficient green chemistry methods employing natural reducing, capping, and stabilizing agents to prepare silver nanoparticles with desired morphology and size have become a major focus of researchers. Biological methods can be used to synthesize silver nanoparticles without the use of any harsh, toxic and expensive chemical substances [51]. The bioreduction of metal ions by combinations of biomolecules found in the extracts of certain organisms (*e.g.*, enzymes/proteins, amino acids, polysaccharides, and vitamins) is environmentally benign, yet chemically complex. Many studies have reported successful synthesis of silver nanoparticle using organisms (microorganisms and biological systems) [52].

#### **2.2.3.1 Synthesis of silver nanoparticles by bacteria**

It was reported that highly stable silver nanoparticles (40 nm) could be synthesized by bio-reduction of aqueous silver ions with a culture supernatant of nonpathogenic bacterium, *Bacillus licheniformis* [53]. Moreover, well-dispersed silver nanocrystals (50 nm) were synthesized using the bacterium *Bacillus licheniformis* [54]. Saifuddin *et al.* [55] have



described a novel combinational synthesis approach for the formation of silver nanoparticles using a combination of culture supernatant of *B. subtilis* and microwave irradiation in water. They reported the extracellular biosynthesis of monodispersed Ag nanoparticles (5-50 nm) using supernatants of *B. subtilis*, but in order to increase the rate of reaction and reduce the aggregation of the produced nanoparticles, they used microwave radiation which might provide uniform heating around the nanoparticles and could assist the digestive ripening of particles with no aggregation.

### **2.2.3.2 Synthesis of silver nanoparticles by fungi**

Silver nanoparticles (5-50 nm) were synthesized extracellularly using *Fusarium oxysporum*, with no evidence of flocculation of the particles even a month after the reaction [56]. The long-term stability of the nanoparticle solution might be due to the stabilization of the silver particles by proteins. The morphology of nanoparticles was highly variable, with generally spherical and occasionally triangular shapes observed in the micrographs. Silver nanoparticles have been reported to interact strongly with proteins including cytochrome *c* (Cc). This protein could be self-assembled on citrate-reduced silver colloid surface [57].

### **2.2.3.3 Synthesis of silver nanoparticles by plants**

*Camellia sinensis* (green tea) extract has been used as a reducing and stabilizing agent for the biosynthesis of silver nanoparticles in an aqueous solution in ambient conditions [58]. It was observed that when the amount of *C. sinensis* extract was increased, the resulted nanoparticles were slightly larger and more spherical. Phenolic acidtype biomolecules (e.g., caffeine and theophylline) present in the *C. sinensis* extract seemed to be responsible for the formation and stabilization of silver nanoparticles. Black tea leaf extracts were also used in the production of silver

nanoparticles [59]. The nanoparticles were stable and had different shapes, such as spheres, trapezoids, prisms, and rods. Polyphenols and flavonoids seemed to be responsible for the biosynthesis of these nanoparticles.

### **2.3 Characterization of silver nanoparticles**

Characterization of nanoparticles is important to understand and control nanoparticles synthesis and applications.

Characterization is performed using a variety of different techniques such as Transmission and Scanning Electron Microscopy (TEM, SEM), Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS), X-ray Photoelectron Spectroscopy (XPS), powder X-ray Diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), and UV–Vis spectroscopy [60].

These techniques are used for determination of different parameters such as particle size, shape, crystallinity, fractal dimensions, pore size and surface area. Moreover, orientation, intercalation and dispersion of nanoparticles and nanotubes in nanocomposite materials could be determined by these techniques.

For instance, the morphology and particle size could be determined by TEM, SEM and AFM. The advantage of AFM over traditional microscopes such as SEM and TEM is that AFM measures three-dimensional images so that particle height and volume can be calculated. Furthermore, dynamic light scattering is used for determination of particles size distribution. Moreover, X-ray diffraction is used for the determination of crystallinity, while UV–Vis spectroscopy is used to confirm sample formation by showing the plasmon resonance.

## **2.4 Properties of Ag nanoparticle:**

Some physicochemical properties of Ag NPs, include size (surface area), shape, surface charge and coating, agglomeration, and dissolution rate, are particularly important for determining their biological interactions and impacts. Smaller particles have a larger surface area and, therefore, have greater toxic potential [61].

It is well known that the shape of silver nanostructures can dramatically affect their physical and chemical properties. Frequently utilized silver nanostructures in the biomedical field include silver spherical nanoparticles, nanowires, nanorods, nanoplates, and nanocubes. Previous Studies have shown that biological effects of Ag NPs depend on different surface charges of their coatings, which affect interaction of Ag NPs with living systems [62].

Agglomeration is known to occur with most engineered nanoparticles. It was shown that agglomeration of Ag NPs occurs in culture media and within the cytoplasm and nuclei of HepG2 cells. Dissolution of Ag NPs as a result of surface oxidation leads to the production of ionic silver. The rate of dissolution depends on the chemical and surface properties of the particle as well as its size, and is further affected by the surrounding media [63].

## **2.5 Silver Nanoparticle Applications**

Silver nanoparticles are of interest due to the unique properties (e.g. size and shape depend on optical, electrical and magnetic properties) which can be incorporated into antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products and electronic components [64]. These particles also have many

applications in different fields such as medical imaging, nano-composites, filters, drug delivery and hyperthermia of tumors [65]. Silver nanoparticles have drawn the attention of researchers due to their extensive applications in areas such as integrated circuits [66] sensors, biolabeling, filters [6], antimicrobial deodorant fibers [67], cell electrodes [68], low-cost paper batteries [69] and antimicrobials [70]. Silver nanoparticles have been used extensively as antimicrobial agents in health industry, food storage, textile coatings and a number of environmental applications.

### **2.5.1 Antibacterial applications**

Silver nanoparticles have important applications in the field of biology such as antibacterial agents and DNA sequencing [71]. Silver has been known to exhibit strong toxicity to a wide range of microorganisms (antibacterial applications). Scientists have long known that silver ions, which flow from nanoparticles when oxidized, are deadly to bacteria [72]. Silver nanoparticles are used just about everywhere, including in cosmetics, socks, food containers, detergents, sprays and a wide range of other products to stop the spread of germs.

### **2.5.2 Medical applications**

One use of silver ion or metallic silver as well as silver nanoparticles can be exploited in medicine for burn treatment, dental materials, coating stainless steel materials, water treatment, and sunscreen lotions [70].

### **2.5.3 Catalysis applications**

Nanocatalysis has recently been a rapidly growing field which involves the use of nanoparticles as catalysts. It is well known that metals such as Au, Ag and Pt and metal ions can catalyzed the decomposition of  $H_2O_2$  to oxygen [73]. It was observed, when the Ag colloid was injected,

chemiluminescence emission from the luminal-  $\text{H}_2\text{O}_2$  system was greatly enhanced [74]. Silver is also the most popular catalyst for the oxidation of ethylene to ethylene oxide and methanol to formaldehyde [74]. Silver nanoparticles immobilized on silica spheres have been tested for their ability to catalyze the reduction of dyes by sodium borohydride ( $\text{NaBH}_4$ ). Catalysis of dyes was chosen as it is easy to detect a change in color when the dyes are reduced. In the absence of silver nanoparticles, the sample was almost stationary showing very little or no reduction of the dyes [75].

#### **2.5.4 Optical applications**

The extraordinary optical properties of noble metal nanoparticles have been taken advantage of optical biosensors and chemosensors. One of research subject focused on the measurement of biological binding signal between antigen and antibody using the triangular Ag-nanoparticles [76]. Optical sensor of zeptomole ( $10^{-21}$ ) sensitivity is another possible application using the potential of silver nanoparticles. Using the surface Plasmon resonance effect, the silver nanoparticles gain a very high sensitivity and the measurements can be conducted in real-time. Silver nanoparticles showed that a peak in extinction, due to the localized surface Plasmon resonance (LSPR) effect. One of the possible applications of the high sensitivity of the LSPR is for in vivo detection. It is possible to monitor the quantity of chemical species inside a cell as well as monitoring the dynamical processes that occur [77].

#### **2.5.5 Electrochemical applications**

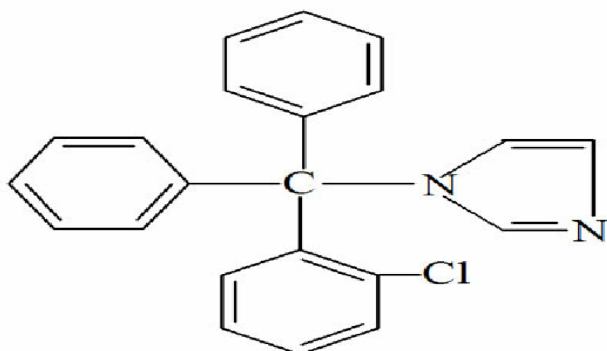
Electrochemical properties of Ag NPs incorporated them in nanoscale sensors that can offer faster response times and lower detection limits. For instance, electrodeposited Ag NPs onto alumina plates gold micropatterned electrode that showed a high sensitivity to hydrogen Peroxide [63].

## 2.6 Silver nanoparticles and their incorporation into antibiotics

Antibiotic molecules may differently interact with Ag NPs, leading to differences in synergistic effects. Although there are limited number of studies exploring combinations of silver and antimicrobials, the topic has recently come into focus. In addition, AgNPs has excellent properties of conformational entropy in polyvalent binding, which make it easy to attach to flexible functional groups of antibiotics [13].

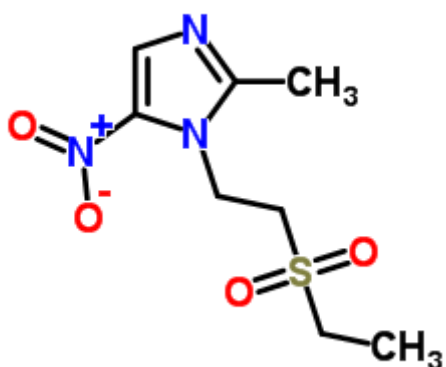
Clotrimazole is an imidazole derivative possessing effective antimycotic activity. Clotrimazole is chemically designated as 1-[(2-chlorophenyl)(diphenyl)methyl]-1*H*-imidazole (Fig.2.2),the formula is  $C_{22}H_{17}ClN_2$  and molar mass is 344.837g/mol. It is used for the prevention of skin and vaginal infections due to dermatophytes and yeasts. The drug clotrimazole has broad-spectrum activity against fungal strains that cause peripheral fungal infections [78]

Clotrimazole was discovered in 1969. Common side effects when taken by mouth include nausea and itchiness. When applied to the skin common side effects include redness and burning. In pregnancy, use on the skin or in the vagina is believed to be safe. There is no evidence of harm when used by mouth during pregnancy but this has been less well studied. When used by mouth, greater care should be taken in those with liver problems. It is in the azole class of medications and works by disrupting the cell membrane [79].



**Fig2.3 Clotrimazole chemical structure**

Tinidazole is structurally similar synthetic imidazole derivative (fig.2.3), having chemical formula  $C_8H_{13}N_3O_4S$ , molar mass 247.273g/mol and widely used as antimicrobials against several infections such as infection of intra-abdominal, respiratory tract, skin, central nervous, oral and dental, bone and joint. Tinidazole is widely prescribed in bacterial vaginosis caused by *Gardnerella vaginalis*. Tinidazole enter into bacterial cell, and convert into active nitroso free radical form either in cytoplasm of the bacteria or in specific organelles of protozoa [80].



**Fig2.4 Tinidazole chemical structure**

## 2.7 Mechanisms of antibacterial effects of silver nanoparticles:

Silver nanoparticles have been extensively used as antimicrobial agents in health industry, food storage, textile coatings and a number of environmental applications [81]. Antimicrobial properties of silver nanoparticles caused the use of these nanometals in different fields of medicine, various industries, animal husbandry, packaging, accessories, cosmetics, health and military. Silver nanoparticles show potential antimicrobial effects against infectious organisms, including *Escherichia coli*, *Bacillus subtilis*, *Vibria cholera*, *Pseudomonas aeruginosa*, *Syphillis typhus*, and *S. aureus* [82]. For instance, it was shown that silver nanoparticles mainly in the range of 1-10 nm attached to the surface of *E. coli* cell membrane, and disturbed its proper function such as respiration and permeability. It was observed that silver nanoparticles had a higher antibacterial effect on *B. subtilis* than on *E. coli*, suggesting a selective antimicrobial effect, possibly related to the structure of the bacterial membrane [83].

Silver nanoparticles have been developed as a potent antibacterial, antifungal [84], antiviral, and anti-inflammatory agent. People have utilized silver nanoparticles since ancient times for medicinal purposes as well as for their attractive physical, chemical, and biological properties. Compared with other metals, silver nanoparticles show higher toxicity to microorganisms while exhibiting lower toxicity to mammalian cells [85]. To date, the most promising applications have been shown in the medical or pharmacological fields, such as biosensors and wound treatment for infection. Silver nanoparticles are available as an antimicrobial gel formulation for conventional topical antimicrobial agents, especially for burn treatment. However, conventional gels and ointments have certain disadvantages, such as the need for a special application syringe to deliver



the gel, which may soften or liquidate unpredictably, and move away from the treatment site rapidly. The gel would not reside on the required region for a long time, resulting in poor patient compliance and unpredictable therapeutic effects [86].

In the past decades, considerable attention has been focused on the development of novel and controlled-release drug-delivery systems to provide a long-term therapeutic concentration of drugs following a single dose [87].

Previous studies also showed that there are several mechanisms about the bactericidal effect of silver nanoparticles [88]. Silver nanoparticles may attach to the surface of the cell membrane, interrupting permeability and metabolic pathways of the cell [89]. Silver nanoparticles not only interact with the surface of the membrane, but can also penetrate into the bacterial cell membrane [90]. In addition, silver nanoparticles can bind to the DNA inside the bacterial cells, preventing its replication or interaction with the bacterial ribosome. It has been discovered that silver nanoparticles can damage the structure of the bacterial cell membrane and reduce the activity of some membranous enzymes, which cause *E. coli* bacteria to die eventually [91].

| <b>Mechanisms of Antibacterial Effects of Ag Nanoparticles</b>   |
|--|
| Cell death due to uncoupling of oxidative phosphorylation  |
| Cell death due to induction of free radical formation  |
| Interference with respiratory chain at cytochrome C level<br>Interference with components of microbial electron transport system |
| Interactions with protein thiol groups & membrane bound enzymes  |
| Interaction with phosphorous- and sulfur-containing compounds<br>such as DNA   |

**Table.2.1 Mechanisms of antibacterial effects of silver nanoparticles**

### **2.8 Toxicity of Ag nanoparticles**

Nano-sized particles can pass through biological membranes and penetrate even very small capillaries throughout the body (e.g., pass through blood-brain and blood testes barriers). Size, morphology, and surface area are recognized as important determinants for toxicity of nanoparticles [92]. Information on the toxicological implication of silver nanoparticles is limited [93]. Toxicity of silver nanoparticles is mostly determined in vitro with particles ranging from approximately 1-100 nm. The in vitro and in vivo toxicity studies revealed that silver nanoparticles have the potential ability to cause chromosomal aberrations and DNA damage, to enter cells and cause cellular damage, and are capable of inducing proliferation arrest in cell lines of zebra fish [92]. Limited health effects of the use of

nanosilver particles in humans have been reported, such as argyria (a condition in which the skin becomes blue or bluish-grey colored) which was appeared to occur only after intake of large amounts of colloidal silver particles (the suspension with nano-silver of different sizes). Several cross-sectional studies reported that argyria is the most frequent adverse outcome from exposure to silver nanoparticles. For instance, prolonged ingestion of colloidal silver can change the color of skin and cause blue-grey appearance on face (the symptoms of argyria) [94]. Silver nanoparticles can bind to different tissues (bind to proteins and enzymes in mammalian cells) and cause toxic effects, such as adhesive interactions with cellular membrane and production of highly reactive and toxic radicals like reactive oxygen species (ROS), which can cause inflammation and show intensive toxic effects on mitochondrial function. Potential target organs for nano-silver toxicity may involve liver and immune system. Accumulation and histopathological effects were seen in livers from rats systemically exposed to silver nanoparticles (10-15 nm). Furthermore, increased liver enzymes were reported in Sprague-Dawley rats after Twenty-eight-day oral exposure to nano-silver particles (60 nm) [95]. It was reported that silver-coated dressing acticoat caused raised liver enzymes and argyria-like symptoms in burn patients [96]. The *in vitro* and *in vivo* studies revealed toxicity effects of silver nanoparticles on immune system, especially cytokine excretion. Application of 1% nano-silver cream (silver nanoparticles with size range of approximately 50 nm), inhibited DNB-induced allergic contact dermatitis, and accumulation in the spleen has also been reported [97]. It has been suggested that silver nanoparticles are especially effective at inhibiting inflammations and may be used to treat immunologic and inflammatory diseases [98]. Oral administration of silver nanoparticles (60 nm) to rats induced some local inflammatory effects [99]. Moreover, in one *in vitro* study, toxicity effects of nano-silver particles on

erythrocytes was reported [100], while an increase in red blood cells was seen after oral administration of silver nanoparticles (60 nm) [99], but not after inhalation of silver nanoparticles (15nm) [92]. An *in vitro* study suggests that nano-silver particles may have cytotoxic effects on mammalian (mouse) spermatogonial germ line stem cells [101]. Silver nanoparticles (10  $\mu\text{g ml}^{-1}$  and above concentration) showed dramatic changes such as necrosis and apoptosis of the cells and at 5-10  $\mu\text{g ml}^{-1}$  drastically reduced mitochondrial function and cell viability. It was reported that there is no evidence available to demonstrate that silver is a cause of neurotoxic damage even though silver deposits have been identified in the region of cutaneous nerves [102]. The respiratory system seemed relatively unaffected by exposure to nano-silver *in vivo* in a 28 days study [92]. However, cytotoxic effect of nano-silver particles on alveolar macrophages and alveolar epithelial cells was demonstrated, *in vitro* [103,104]. In a 90 days inhalation study, decrease in lung functions, including tidal volume, minute volume and peak inspiration flow as well as inflammatory lesions in lung morphology and effects on inflammatory markers were reported [105]. Kim et al. (2008) [95] performed a bone marrow micronucleus test as a part of a 28-day oral administration study in order to investigate genotoxic effects or carcinogenicity of exposure to silver nanoparticles. No significant genotoxic potential of oral exposure to silver nanoparticles (60 nm) was found in this study. An increase in (mostly local) malignant tumors was found following chronic subcutaneous administration of high doses colloidal silver [106].

## **2.9 Antibiotic Nanoparticle Complex ( Nanoplex)**

Silver nanoparticles in combination with antibiotics has been used previously, and there are many efforts have been made to overcome the emerging problem of antibiotic resistance among a variety of disease

causing bacteria and advances in the field of nanobiotechnology may offer a great opportunity of research in this field. Therefore, studies on combination of antibiotic agents and synthetic and natural organic or inorganic nanomaterials are of great importance. There are thousands of papers published on nanotechnology and specifically on nanoparticles synthesis and their probable practical applications during recent decades.

Li et al. [107], synthesized silver nanoparticles by reducing  $\text{AgNO}_3$  aqueous solution with ascorbic acid aqueous solution with an average size of 20 nm. Exposing *E. coli* to these nanoparticles to a combination with the beta lactam antibiotic amoxicillin increased the bactericidal efficiency when compared to Ag NPs or amoxicillin alone. In another study Shahverdi et al. [5], the combinations of Ag NPs with five antibiotics (penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin) were tested against both model microbes *E. coli* and *S. aureus*. Ag NPs were produced by aqueous Ag(I) reduction from the supernatant of a *Klebsiella pneumonia* culture and their sizes was between 5–30 nm. In general, enhanced killing of the bacteria was recorded, when a combinational treatment was employed (Ag NPs + Antibiotic). Notably, the most prominent effects were observed for vancomycin, amoxicillin and penicillin G against *S. aureus*.

Another study showed that AgNPs in combination with antibiotics have better antibacterial effect as compared with AgNPs alone and hence can be used in the treatment of infectious diseases caused by bacteria. The maximum effect, with a 17.8-fold increase in inhibition zone, was observed for amoxicillin with AgNPs against *S. marcescens* proving the synergistic role of AgNPs. Therefore, it may be used to augment the activities of antibiotics [108].

Wang. L et al; [109] demonstrate that antibiotic-linkage to surface-functionalized silica nanoparticles (SNP) significantly enhances their effectiveness against *Escherichia coli*, and *Staphylococcus aureus*, and even methicillin-resistant *S. aureus* (MRSA) strains that are resistant to most antibiotics. The commonly-used antibiotic Penicillin-G (PenG) was complexed to dye-labeled sNPs (15 nm diameter) containing carboxyl groups located as either surface-functional groups, or on polymer-chains extending from surfaces. Both sNPs configurations efficiently killed bacteria, including MRSA strains.

Another study showed preparation and characterization of antibiotic nanoparticle complex (or nanoplex) by self-assembly amphiphile-polyelectrolyte complexation process. Ofloxacin (OFX) and levofloxacin (LEV) are used as the antibiotics with dextran sulfate (DXT) as the polyelectrolyte. The nanoplex possesses high drug loading (up to 80%) and size < 400 nm ideal for sputum penetration [110].

In another study, the combination effects between nano-Ags and the conventional antibiotics ampicillin, chloramphenicol and kanamycin against various pathogenic bacteria were investigated. The MIC and fractional inhibitory concentration index (FICI) were determined to confirm antibacterial susceptibility and synergistic effects. The results showed that nano-Ags possessed antibacterial effects and synergistic activities. The antibiofilm activities of nano-Ags alone or in combination with antibiotics were also investigated. Formation of biofilm is associated with resistance to antimicrobial agents and chronic bacterial infections. The results indicated that nano-Ags also had antibiofilm activities [111]

The main aim in another study is to synthesize the silver nanoparticles chemically and study their synergistic effect with the commercially

available antibiotics on clinical isolates of multi-drug resistant bacteria. The silver nanoparticles were synthesized chemically using sodium citrate and the formation of silver nanoparticles was checked using UV-Vis Spectrophotometer, the peak at 420nm confirms the synthesis of silver nanoparticles. Various concentrations of silver nanoparticles conjugates were used to check the survival kinetics of the bacteria. It was observed that there was a 7 to 9 log decrease in bacterial cells when silver nanoparticles conjugated with antibiotics was used [112].

In another study a systematic study quantifying the synergistic effects of antibiotics with different modes of action and different chemical structures in combination with AgNPs against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was performed. Employing the microdilution method as more suitable and reliable than the disc diffusion method, strong synergistic effects were shown for all tested antibiotics combined with AgNPs at very low concentrations of both antibiotics and AgNPs. No trends were observed for synergistic effects of antibiotics with different modes of action and different chemical structures in combination with AgNPs, indicating non-specific synergistic effects. Moreover, a very low amount of silver is needed for effective antibacterial action of the antibiotics, which represents an important finding for potential medical applications due to the negligible cytotoxic effect of AgNPs towards human cells at these concentration levels [113].

Another study based on the synergistic effect of Cephalexin antibiotic with AgNP. The nanoparticles were evaluated for their increased antimicrobial activities with Cephalexin antibiotic against *E.coli* and *S. aureus*. The antibacterial activity of Cephalexin was increased in the presence of AgNPs against test strains. The results showed that the combination of antibiotics with AgNPs have better antimicrobial effects [114].

In another study the bactericidal action of silver (0) nanoparticles and amoxicillin on *Escherichia coli* is studied, respectively. Increasing concentration of both amoxicillin (0–0.525 mg ml<sup>-1</sup>) and silver nanoparticles (0–40 µg ml<sup>-1</sup>) showed a higher antibacterial effect in Luria–Bertani (LB) medium. *Escherichia coli* cells have different bactericidal sensitivity to them. When amoxicillin and silver nanoparticles are combined, it results in greater bactericidal efficiency on *Escherichia coli* cells than when they were applied separately. Dynamic tests on bacterial growth indicated that exponential and stationary phases are greatly decreased and delayed in the synergistic effect of amoxicillin and silver nanoparticles. In addition, the effect induced by a preincubation with silver nanoparticles is examined. The results show that solutions with more silver nanoparticles have better antimicrobial effects [115]



## Chapter Three

### Materials and methods

#### 3.1 Materials

Silver nitrate ( $\text{AgNO}_3$ ) (99.9%, Tianjin Bodi Chem. Co. Ltd.); tri-sodium citrate (99.8%, Tianjin Bodi Chem. Co. Ltd.); Clotrimazole and Tindiazole, in their pure forms were received from Amipharma, Khartoum, Sudan; deionized water was used in all solution preparations.

#### 3.2 Methods:

##### 3.2.1 Synthesis of Ag nanoparticle by chemical reduction

The silver nanoparticles were prepared using chemical reduction method. 0.1 gm of  $\text{AgNO}_3$  was dissolved in 500 ml deionized water, the solution was heated to boiling, 10 ml of 1% tri-sodium citrate were added to 500 ml of  $\text{AgNO}_3$  after boiling, and mixed vigorously. The mixture was left on a hot plate at 90C for 2 hours, cooled to room temperature; the solution color was yellowish green.

#### 3.3 Characterization of silver nanoparticle

After completion of the synthesis of silver nanoparticle, several characterization techniques were used to investigate the morphology, structure, surface area, chemical bonding.

Formation of AgNPs was confirmed by Ultraviolet-visible spectral analysis. The surface morphology and size of the AgNPs were examined using a Transmission electron microscope (TEM). In this chapter, all, these characterization techniques are briefly described.

### 3.3.1. Ultra violet- visible spectroscopy (UV)

The absorbance spectra were recorded using Ultraviolet-visible spectroscopy (UV-1800 Shimadzu UV spectrophotometer) at a wavelength of 400–800 nm.

Typically, to exclude the background absorption spectrum, equipment was first calibrated with a blank sample of the chosen solvent. The sample is placed into the cuvette and is held in the spectrophotometer sample chamber. A pulse of wide-spectrum light, generated by a tungsten bulb, is passed through the sample and the resulting absorption is collected and the blank sample is subtracted out.

The absorption phenomenon shown by the nanoparticles is due to surface Plasmon resonance. The optical absorption spectra of metal nanoparticles are dominated by surface Plasmon resonance, which shift to longer wavelengths with increasing particle size.



**Fig (3.1): UV-visible spectroscopy**

### 3.3.2. Transmission electron microscopy (TEM)

Sample was prepared by placing a drop of the nanoparticle solution onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 45 min. TEM measurements were performed on a 'Philips CM200' instrument operated at an accelerating voltage of 120 kV. The sizing of the samples was carried out from transmission electron micrographs using the software Image Tool for Windows (Version 2.0), while data were analyzed by means of the software Microcal Origin 6.0.



**Fig (3.2): Transmission electron microscopy (TEM)**

### 3.3.3. Fourier transforms infrared spectroscopy (FTIR)

Fourier transform infrared spectra of complexes of antibiotics with Ag nanoparticles were obtained using a Nicolet 6700 FT-IR spectrometer at room temperature in the  $4000 - 400 \text{ cm}^{-1}$  wavenumber range, with a 4

cm<sup>-1</sup> resolution. Before each analysis, a pellet made of a mixture of specimens powder and KBr were pressed and dried overnight at 105oC.



**Figure.3.3. Fourier transforms infrared spectroscopy FTIR (Nicolet 6700)**

### 3.4. Assay for antibacterial activity

#### 3.4.1 Test organisms

The test organisms (selected strains from four bacteria) were kindly provided by Stack laboratory, Sudan.

**Table 3.1 References strains of bacteria**

|                               |            |                       |
|-------------------------------|------------|-----------------------|
| <i>Bacillus subtilis</i>      | NCTC 8239  | (Gram positive rods)  |
| <i>Staphylococcus aureus</i>  | ATCC 25923 | (Gram positive cocci) |
| <i>Escherichia coli</i>       | ATCC 25922 | (Gram negative rods)  |
| <i>Pseudomonas aeruginosa</i> | ATCC 27853 | (Gram negative rods)  |

ATCC is the American Type Culture Collection, Rockville, Maryland, U.S.A.

### **3.4.2 Culture media**

#### **3.4.2.1 Nutrient broth**

Culture medium contained peptone, yeast extract and sodium chloride. It was prepared according to (Barrow and Feltham, 1993) [116], by dissolving 13 gram of the medium in one litre of distilled water. The pH of the medium was adjusted to 7.4 and the medium was then distributed into screw capped bottles, 5 ml each and sterilized by autoclaved at 121°C for 15 minutes.

#### **3.4.2.2. Nutrient agar**

The medium contained lab- lemco powder (1.0g), yeast extract (2.0g), peptone (5.0g) and agar No.3 (15.0g). Twenty-eight grams of dehydrated medium were dissolved in one litre of distilled water and the pH was adjusted to 7.4. The dissolved medium was sterilized by autoclaving at 121°C for 15 minutes.

#### **3.4.2.3. Mueller Hinton agar**

Thirty-eight grams of Mueller Hinton agar powder were weighed, dissolved in 1 litre of distilled water and allowed to soak for 10 minutes. The medium was placed in water bath to dissolve, swirled to mix and sterilized by autoclaving for 15 minutes at 121°C, cooled to 47°C mixed well then poured into sterile Petri dishes.

### **3.4.3. Disc diffusion method**

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of

chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a “zone of inhibition”.

The paper disc diffusion method of [117] was adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. Twenty ml aliquots of the molten Mueller Hinton agar were distributed into sterile Petri-dishes.

About 0.1 ml of the standardized bacterial stock suspension  $10^8 - 10^9$  C.F.U/ ml were streaked on Mueller Hinton agar medium plates using sterile cotton swab. Sterilized filter paper disc (6 mm diameter) were soaked in the prepared extracts, and then were placed on surface of the test bacteria plates. The plates were incubated for 24 h and the diameters of the inhibition zones were measured. Reference drugs and 10% Dimethyl sulfoxide (DMSO) were used as the positive and negative controls, respectively. After incubation period, the diameters of the resultant growth inhibition zone were measured.

### **3.5. Combination of Tindiazole and clotrimazole with Silver nanoparticles**

Silver nanoparticles /Tinidazole or Clotrimazole complexes were prepared by mixing the two substances (1ml:0.5 mg) which followed by an incubation step for overnight at room temperature and then used to treat the *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*.

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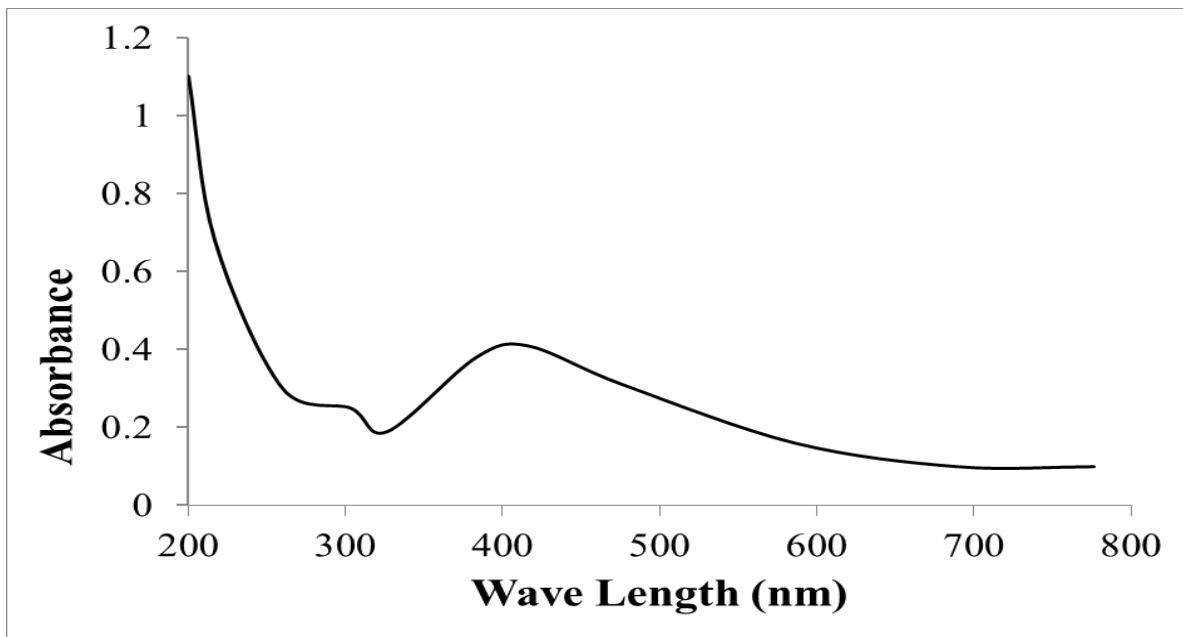
## Chapter Four

### Results and discussion

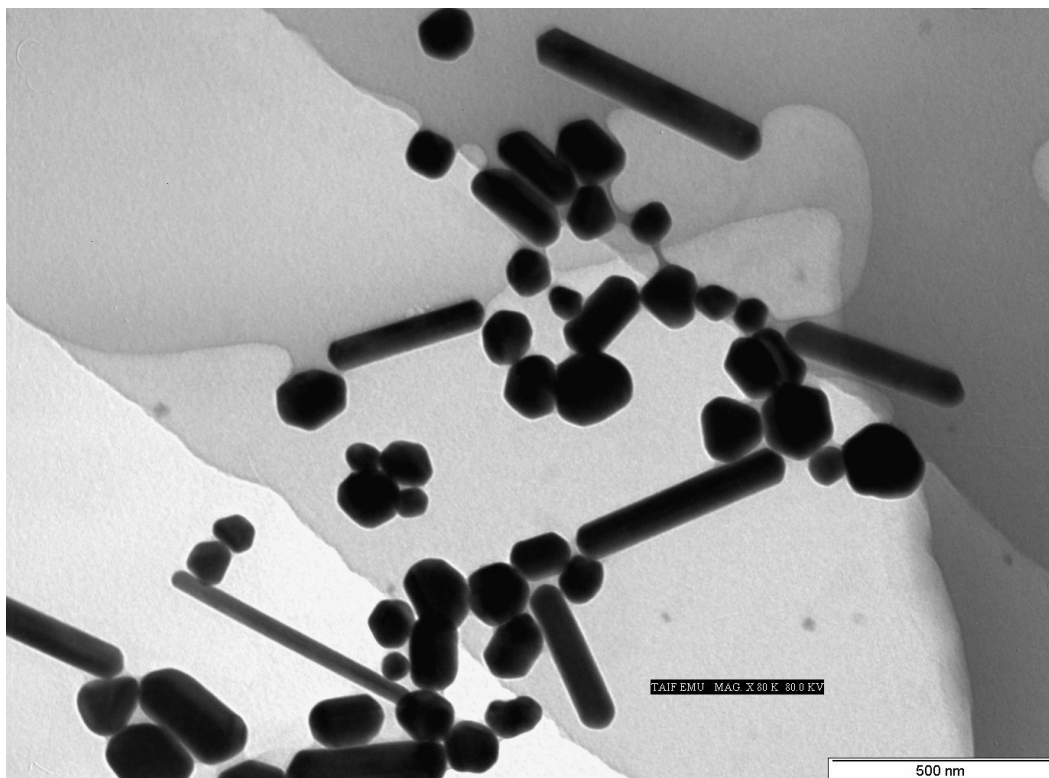
#### 4.1 Synthesis and characterization of Ag nanoparticle

Silver nanoparticles were synthesized according to the method described in the previous section, the solution turned pale yellow indicating that the silver nanoparticles were formed. It was noted that a yellow colour was developed in solution, which has been attributed to the excitation of surface plasmon resonance (SPR) in Ag NPs. The prepared aqueous solution of Ag nanoparticles showed an absorption band at 415 nm as shown in Figure 4.1. The SPR arising due to collective oscillation of free conduction electron induced by an interacting electromagnetic field [118]. Observation of strong but broad surface plasmon peak has been well documented for various Me-NPs, with sizes ranging all the way from 2 to 100 nm [5,119]. It was found that the silver nanoparticles show SPR peak at around 420 nm [120]. Previous study also shows that the hexagonal nanoplates have a strong SPR absorption peak at about 418 nm [121].

Furthermore, TEM analysis was performed to study the size and shape. TEM micrograph of the prepared solution of silver nanoparticles is shown in the **Figure 4.2**. In the TEM analysis, silver nanoparticles were hexagonal and rods-like in shape with size 50 and 55 nm respectively. The formation of these shapes is due to aggregation during store of solution. Previous study showed TEM photographs indicate that the nanopowders consist of well dispersed agglomerates of grains with a narrow size distribution (40 and 60 nm) this is in a good agreement with our present findings [122].



**Figure 4.1: The UV absorption spectra of silver nanoparticles**



**Figure 4.2: TEM micrographs of Ag NPS**



## 4.2. Fourier transforms infrared spectroscopy (FTIR)

Functional groups present on the chemical structure of compounds give characteristic vibrational peak (stretching, bending etc.) on FT-IR spectra, which is unique for that particular functional group. These vibrational peaks interpreted for structural characterization of test compounds.

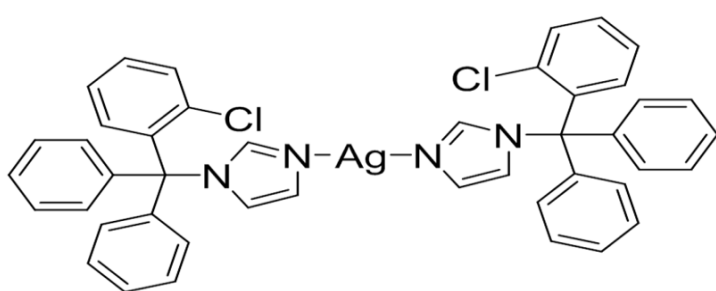
The infrared spectra of clotrimazole and Tinidazole are shown in Figures 4.3 and 4.5 respectively, and the spectra of the AgNPs incorporated antibiotics are shown in Figures 4.4 and 4.6.

FT-IR spectra of clotrimazole are shown in Figure 4.3, the peak at  $3059\text{ cm}^{-1}$  is assigned to C-H stretching vibration of  $\text{sp}^2$  hybridized carbon atom (aromatic ring). The absorption peaks at 1483 and 1594 could be attributed to stretching vibrations of  $\text{-C=C}$  and  $\text{-C=N}$  groups.  $\text{-CH}$  bending vibrations and  $\text{-C—C}$  stretching vibration have given peaks at 1439 and  $1314\text{ cm}^{-1}$  respectively. The spectrum has also shown a number of bands lying in the range 900 to  $600\text{ cm}^{-1}$  that could be attributed to  $\text{-CH}$  out of plane vibration of the aromatic ring and  $\text{-CH}$  rocking vibration.

The FT-IR spectrum of tinidazole exhibited two sharp vibrational peaks at 2957 and  $2913\text{ cm}^{-1}$  that was assigned to C-H stretching vibration for  $\text{sp}^3$  hybridized carbon atoms. The IR peak at  $1760\text{ cm}^{-1}$  were attributed to  $\text{C=N}$  (imidazole ring). The absorption peaks at 1523, 1455 and  $1373\text{ cm}^{-1}$  were attributed to  $\text{-CH}_2$ ,  $\text{-C—C}$  stretching vibration and  $\text{-CH}_3$  bending vibrations, respectively. The IR peaks appeared at 1300, 1259, 1190, 1120, 1033 and  $1026\text{ cm}^{-1}$  were assigned to  $\text{S=O}$  asymmetric stretching,  $\text{C—O}$  stretching,  $\text{N=O}$  stretching,  $\text{S=O}$  symmetric stretching  $\text{C—N}$  stretching, and  $\text{-CH}$  in-plane bending for aromatic system, respectively. The two peaks around 3003 and  $3130\text{ cm}^{-1}$  possibly due to  $\text{-CH}$  stretching vibration of  $\text{sp}^2$  hybridized system (aromatic ring). Finally, many small sharp peaks

appeared between  $900\text{-}600\text{ cm}^{-1}$  that could be assigned to  $\text{-CH}$  out of plane vibration of the aromatic ring and  $\text{-CH}$  rocking vibration.

The infrared spectrum of Clotrimazole exhibited a characteristic peak of  $\text{C}=\text{N}$  at  $1594\text{ cm}^{-1}$ , which showed significant shifts to  $1585\text{ cm}^{-1}$  in the infrared spectra of the Clotrimazole+AgNPs complexes, which confirmed the participation of the  $\text{C}=\text{N}$  group in the complexation process.



**Fig 4.3 Expected Clotrimazole/Ag NPs complexes**

The infrared spectrum of Tinidazole exhibited a characteristic peak of  $\text{C}=\text{N}$  at  $1760\text{ cm}^{-1}$ , which showed significant shifts to  $1621\text{ cm}^{-1}$  in the infrared spectra of the Tinidazole+Ag NPs complexes, which confirmed the participation of the  $\text{C}=\text{N}$  group in the complexation process.

A comparison of the relevant IR spectral bands of clotrimazole and tindiazole with the silver nanoparticle indicated shifts in the frequencies as well as reductions in the band intensities, which confirm the formation of charge transfer complexes. These shifts in the values were due to changes in the electronic structures and molecular symmetries of reactants upon complex formation.

Overall, the FT-IR result suggested that due to enhanced force constant and conjugation effect in some bonds, the chemical stability of respective bonds in complexes and tinidazole and clotrimazole could be enhanced as compared to control.

**Table (4.1) Characteristic FTIR peaks of the Clotrimazole and Tindiazole**

| Name of the compound | Characteristic peaks (cm <sup>-1</sup> ) | Corresponding functional groups |
|----------------------|--|---------------------------------|
| Clotrimazole         | 3059                                     | C-H(st) aromatic                |
|                      | 1439                                     | C-H bending                     |
|                      | 1594                                     | C=N                             |
|                      | 1483                                     | C=C                             |
|                      | 1314                                     | C-C (st) vibration              |
| Tindiazole           | 1301                                     | S=O(asymmetric st)              |
|                      | 1123                                     | S=O (symmetric st)              |
|                      | 1760                                     | C=N(imidazole ring)             |
|                      | 1039                                     | C-N st                          |
|                      | 3000, 3131                               | C-H (st) aromatic               |
|                      | 1265                                     | N-O                             |
|                      | 1026                                     | C-H bending aromatic            |
|                      | 1144                                     | N=O (symmetric st)              |
|                      | 1478                                     | CH <sub>2</sub> (st)            |
| 1428                 | C-C (st)                                 |                                 |

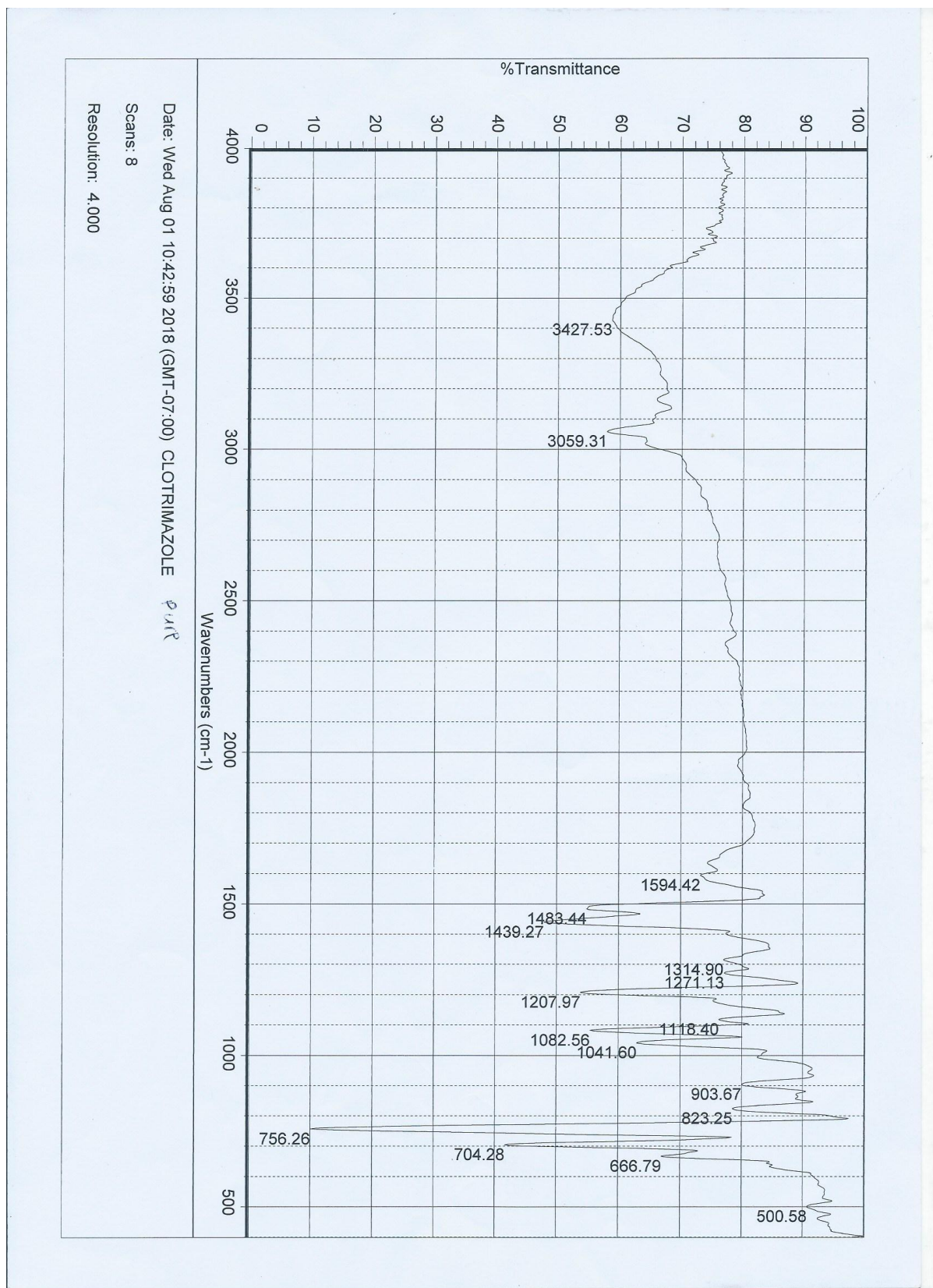


Fig.4.4. Infrared spectra of Clotrimazole

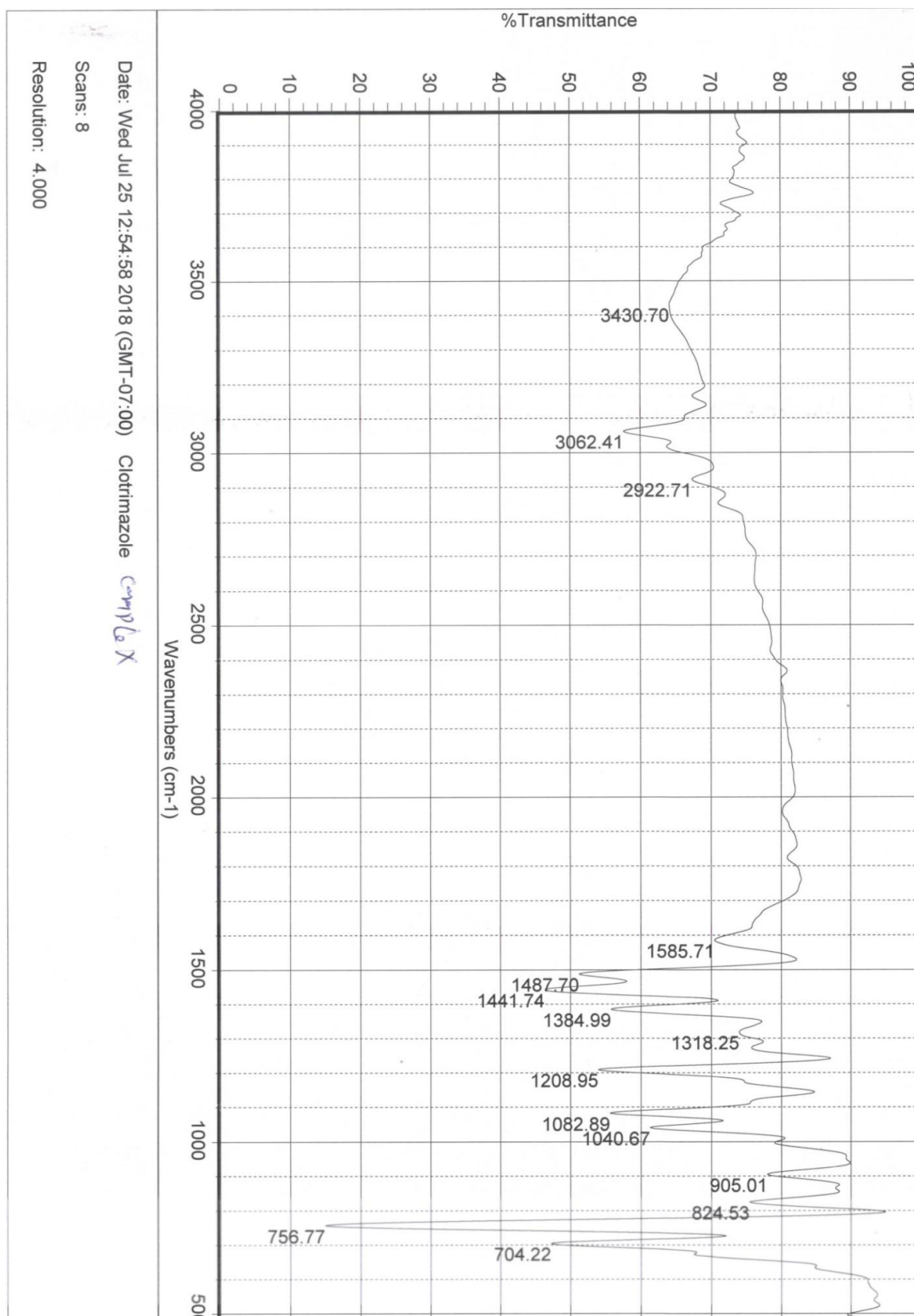
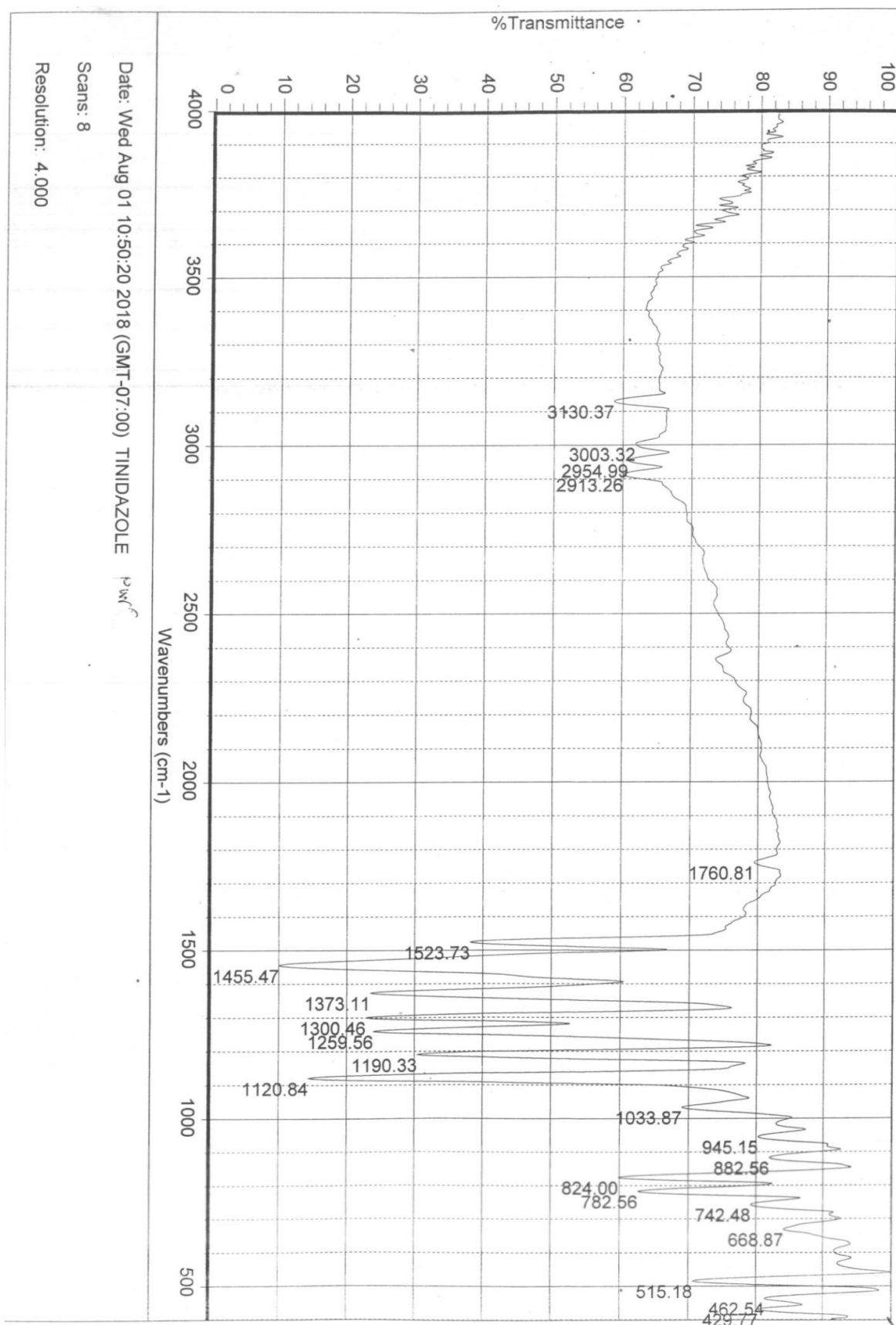
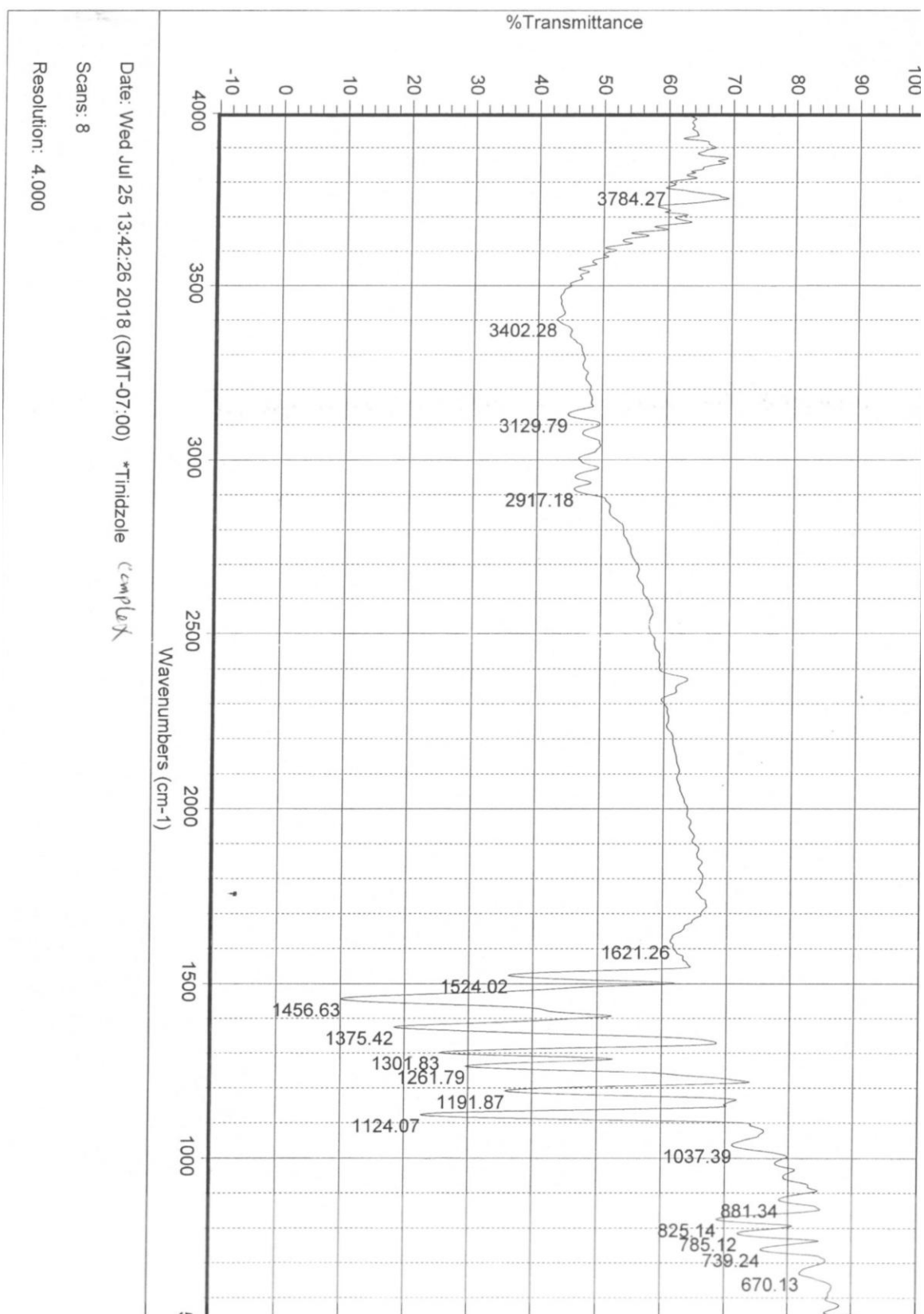


Fig.4.5 Infrared spectra of Clotrimazole/Ag nanoparticles complex



**Fig.4.6 Infrared spectrum of Tinidazole**



**Fig.4.7 Infrared spectrum of Tindiazole/Ag NPs complex**

### 4.3. Assay for antibacterial activity

#### 4.3.1. Antibacterial activity of silver nanoparticles

Silver nanoparticle solution was screened for its antibacterial activity against four standard bacterial species (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The antibacterial assay was conducted by using the disc diffusion method. The tests were carried out at a concentration of 20mg/ml. The zone of inhibition of bacterial growth were measured after 24 hours and the measurements were done (in mm) from the end of the growth of one side of the disc to the beginning of growth of the other side including the diameter of the disc. The means of diameter of the growth inhibition zones obtained in the experiment are shown in Table [4.2]. The results were interpreted in terms of commonly used terms: sensitive, intermediate and resistant. On this basis compounds resulting in greater than 18 mm growth inhibition zones are considered to possess relatively high antibacterial activity, and those resulting in 14-18 mm inhibition zone are of intermediate, and those resulting in zones below 14 mm are low or inactive [123].

The results clearly indicated variation in the activity against different bacterial species. Ag NPs show intermediate activity towards *Pseudomonas aeruginosa* (16mm), *Bacillus subtilis* (15.5±0.7mm), *Escherichia coli* (15mm) and *Staphylococcus aureus* (14.5±0.7) as shown in Fig 4.7, Fig 4.8 and Fig 4.9 respectively.

In present study, zone of inhibition was found to be high (16 mm) against *p. aeruginosa* and low (14.5 mm) against *Staphylococcus aureus* Fig 4.10. The results showed, approximately, similar activity against both gram positive and gram negative bacterial, ranging between 14.5 mm towards *Staphylococcus aureus*, 16 mm towards *p. aeruginosa*.



These findings are in agreement with previous studies that examined antibacterial activity of AgNPs synthesized by chemical reduction method. The results showed reasonable bactericidal activity against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [120]. Other studies [8] showed similar results as those observed in this study. *E. coli* cells were inhibited by Ag NPs at lower concentrations as compared to *S. aureus* (Gram positive). Also, the MIC of Ag NPs was lower when testing against *E. coli* than when testing against *S. aureus*.

In comparison to previous study [31], this solution has slightly high activity towards *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*.

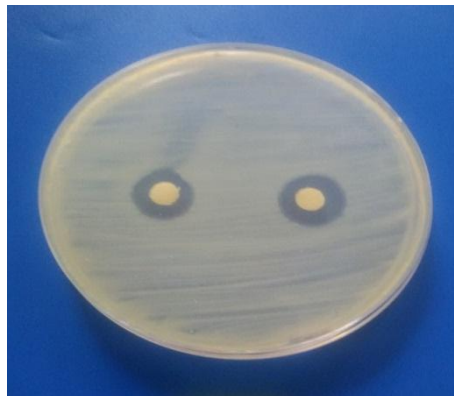
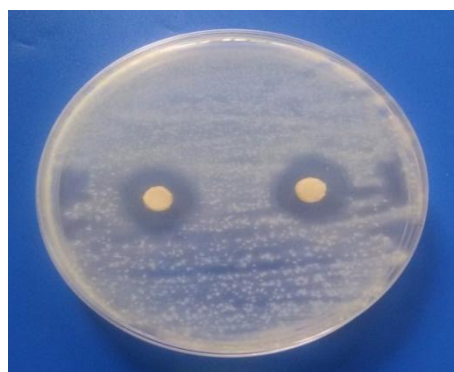
The mechanism of the inhibitory action of the metal nanoparticles on microorganisms is not still clearly known. The antibacterial effect could be explained on the basis of small sized AgNPs synthesized by chemical reduction method that provides better contact and interaction with the bacterial cell than larger ones [124]. In addition, silver ions released from AgNPs may penetrate cell membranes interacting with sulfur and phosphorus containing compounds such as proteins and DNA that may inhibit DNA replication and results in loss of cell viability and ultimately leads to cell death [125]. It has also been proposed that the AgNPs have a sustained release of silver ions once inside the bacterial cells [126], and these ions can interact with thiol groups present in enzymes such as NADH dehydrogenases and disrupt the respiratory chain [127]. The formation of free radicals by AgNPs induces oxidative stress which may be considered to be another mechanism of cell death [128].

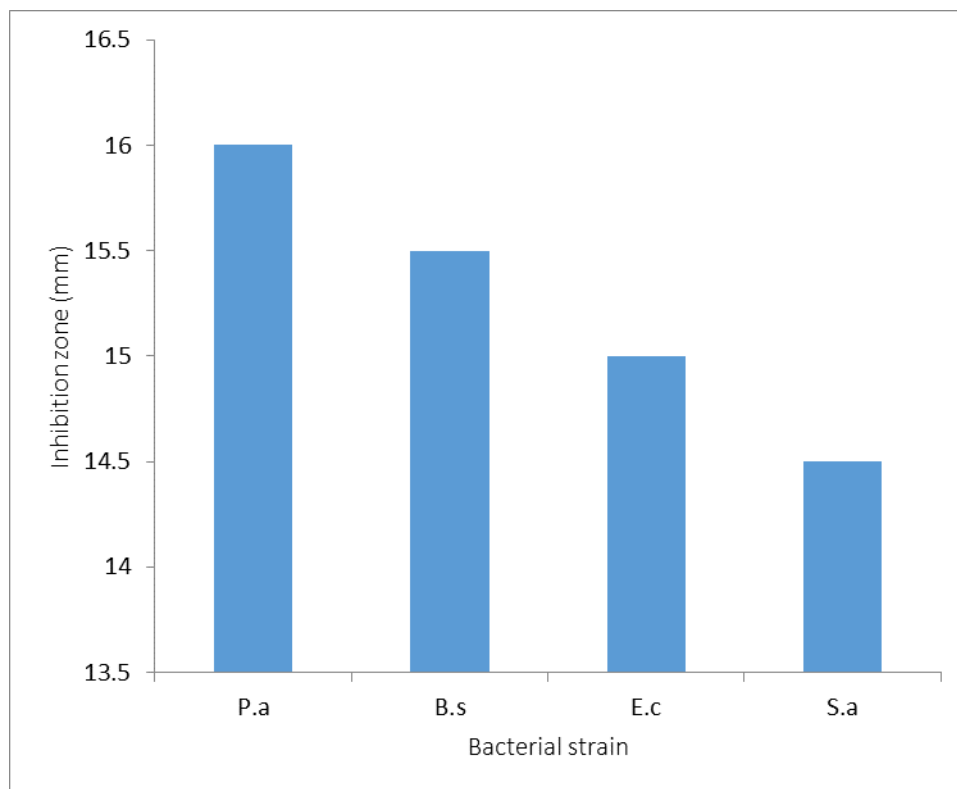
**Table (4.2): Anti-bacterial activity of Ag NPs, antibiotics and complexes against four bacterial strains.**

| Compound                       | Concentration<br>(mg/ml) | Bacteria strain   |            |            |            |
|--------------------------------|--------------------------|-------------------|------------|------------|------------|
|                                |                          | M. D. I. Z. (mm)* |            |            |            |
|                                |                          | <i>B.s</i>        | <i>S.a</i> | <i>E.c</i> | <i>P.a</i> |
| Ag nanoparticle                | 2                        | 15.5±0.7          | 14.5±0.7   | 15±0       | 16±0       |
| Clotrimazol                    | 2                        | 30±0              | 15±1.4     | 17±1.4     | 12.5±0.7   |
| Tindazole                      | 2                        | 11.5±0.7          | 12±1.4     | 19±1.4     | 13±0       |
| Tindazole+Ag<br>nanoparticle   | 2                        | 14±0              | 12.5±0.7   | 12.5±0.7   | 11.5±0.7   |
|                                | 1                        | 15±0              | 14±0       | 13.5±0.7   | 13.5±0.7   |
|                                | 0.5                      | 14±0              | 15±0       | 15±0       | 13±0       |
| Clotrimazol+Ag<br>nanoparticle | 2                        | 13 ±0             | 14±0       | 10.5±0.7   | 13.5±0.7   |
|                                | 1                        | 12.5±0.7          | 15±0       | 12.5±0.7   | 14.5±0.7   |
|                                | 0.5                      | 14±0              | 16±0       | 16.5±0.7   | 13.5±0.7   |
| Control (DMSO)                 | 10%                      | –                 | –          | –          | –          |

\*M. D. I. Z., Mean diameter of growth inhibition zone in mm

**Key:** *B.s* = *Bacillus subtilis*, *S.a* = *Staphylococcus aureus*,  
*E.c*=*Escherichia coli* and *P.a* = *Pseudomonas aeruginosa*.

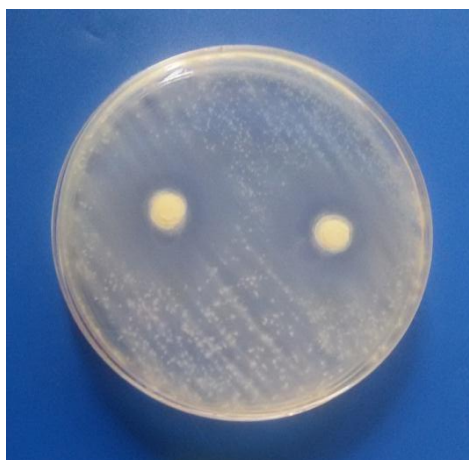
**Fig 4.8 Antibacterial activity of Ag nanoparticles against:****(A) *Staphylococcus aureus*****(B) *Bacillus subtilis*****(C) *Pseudomonas aeruginosa***



**Fig (4.9): Ag nanoparticle growth inhibition zone curve**

### 4.3.2. Antibacterial activity of Clotrimazol and Tindiazole

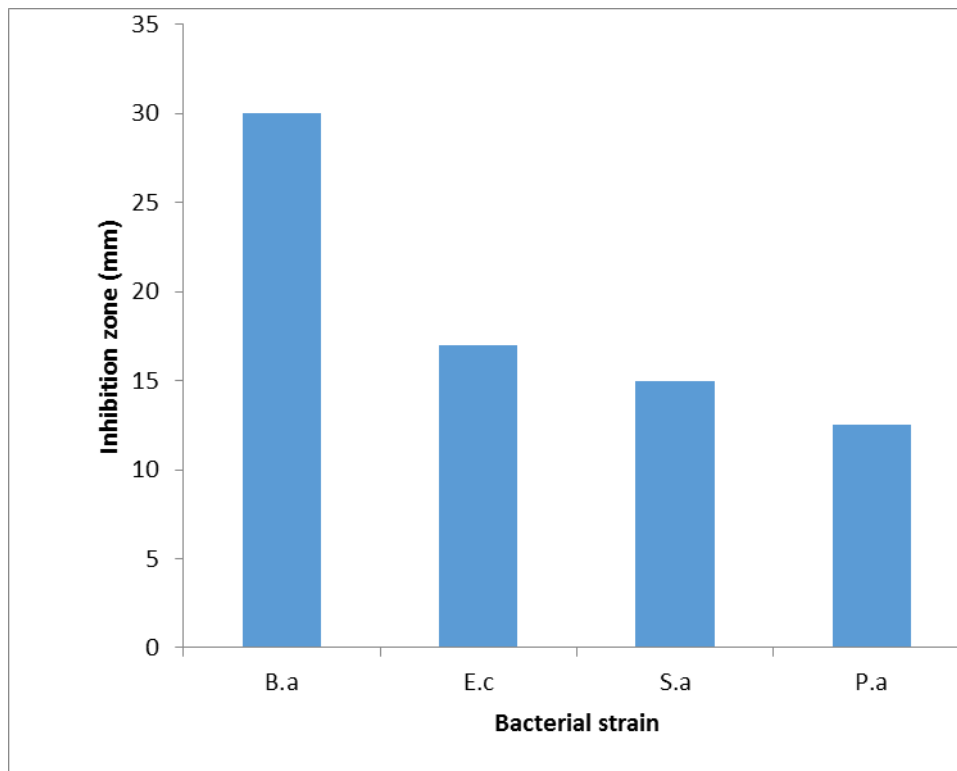
The antibacterial activity Ag NPS of the all tested samples were compared to two standard antibiotics, Clotrimazole and Tindiazole at concentration of 20 meg/disc are showed in Table [4.2]. Clotrimazole showed high activity towards *Bacillus subtilis* (30mm), and Tindiazole showed high activity towards *Escherichia coli* ( $19\pm 1.4\text{mm}$ ) as showed in Figure [4.9, 4.10, 4.11 and 4.12].



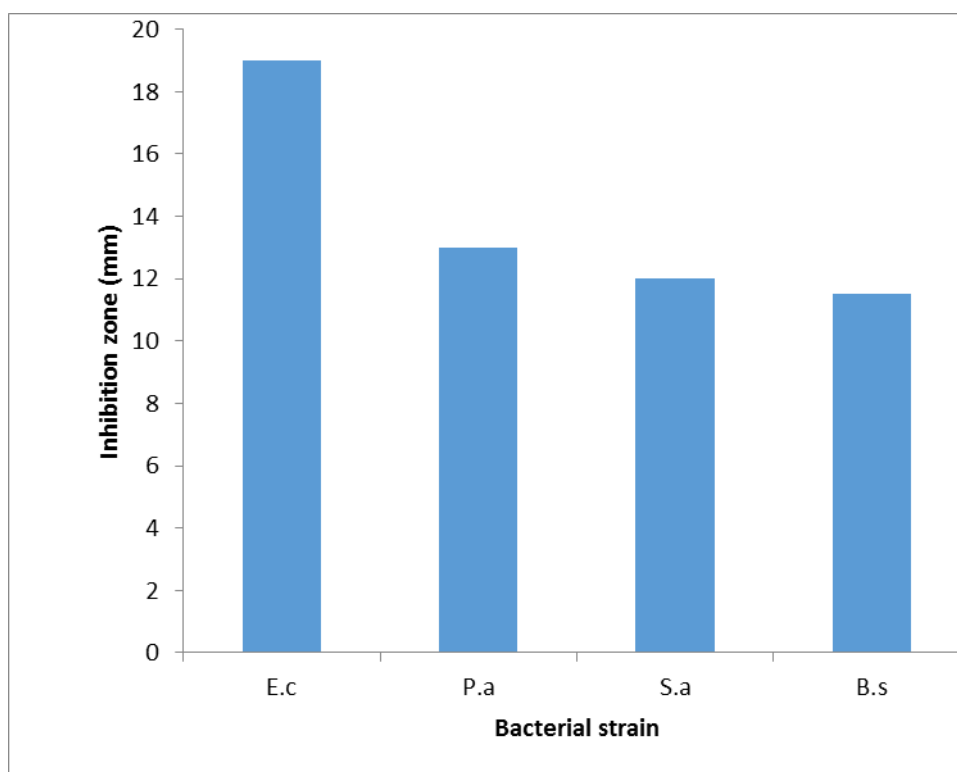
**Fig 4.10** Anti-bacterial activity of Clotrimazol against *Bacillus subtilis*



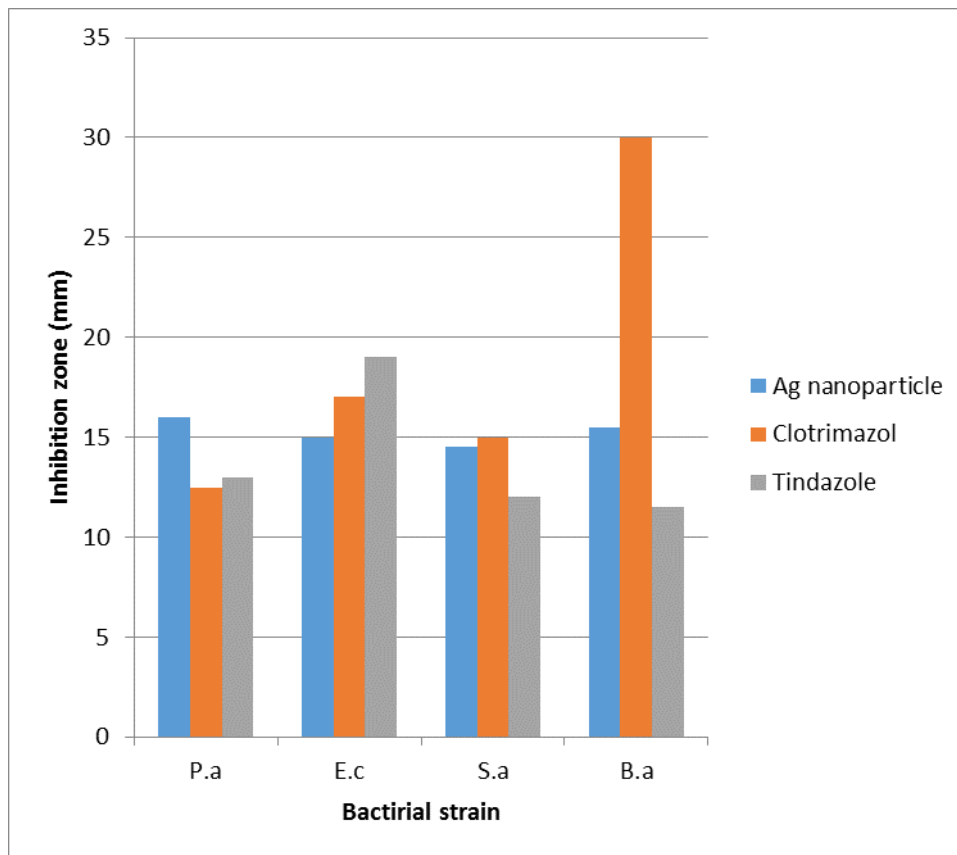
**Fig 4.11** Anti-bacterial activity of Tindazole against *Escherichia coli*



**Fig.4.12: growth inhibition zone curve of Clotrimazole**



**Fig.4.13: growth inhibition zone curve of Tindiazole**



**Fig.4.14: Ag nanoparticle growth inhibition zone of Ag NPs, Tindazole and Clotrimazole**

### 4.3.3. Synergistic Effect of Antibiotics combined with Silver

#### Nanoparticles:

The synergistic effect of AgNPs combined with antibiotics against both Gram-positive and Gram-negative bacteria, namely *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, was evaluated using the disc diffusion method to determine the growth inhibition zones of the used antibiotics combined with AgNPs (Table 4.2). The results show a significant increase in antibacterial activity of antibiotics in the presence of silver nanoparticles.

Clotrimazole + AgNPs show a significant increase in antibacterial activity towards *Pseudomonas aeruginosa* [ $14.5 \pm 0.7$  mm] at concentration 1mg/ml, *Staphylococcus aureus* ( $16 \pm 0$  mm) at concentration 0.5 mg/ml, but there is a decrease in activity after combination towards *Escherichia coli* and *Bacillus subtilis*.

Tindiazole + AgNPs showed a significant increase in antibacterial activity towards *Bacillus subtilis* ( $15 \pm 0$  mm) at concentration 1mg/ml, *Staphylococcus aureus* ( $15 \pm 0$  mm) at concentration 0.5 mg/ml, *Pseudomonas aeruginosa* ( $13.5 \pm 0.7$ ) at concentration 1mg/ml, but there is a decrease in activity after combination towards *Escherichia coli*.

It is concluded that AgNPs have augmented the efficacy of most of the antibiotics, therefore, may be used in combination with antibiotics against drug resistant bacteria.

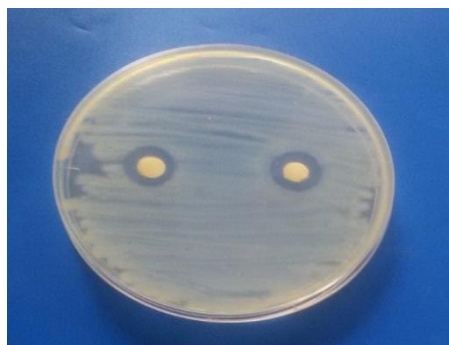
Our results are also in correlation with the work previously done by some researchers studied the synergistic effect of silver nanoparticles alone and in combination with conventional antibiotics against pathogenic strains [129,130,131]



Another study showed that AgNPs in combination with antibiotics have better antibacterial effect as compared with AgNPs alone and hence can be used in the treatment of infectious diseases caused by bacteria. The maximum effect, with a 17.8 fold increase in inhibition zone, was observed for amoxicillin with AgNPs against *S. marcescens* proving the synergistic role of AgNPs. Therefore, it may be used to augment the activities of antibiotics [108].

We conclude that low concentrations of silver nanoparticles with antibiotics have strong effects on the growth of bacteria.

**Fig 4.15. Anti-bacterial activity of Clotrimazol /Ag NPs complexes  
(2mg/ml) against:**

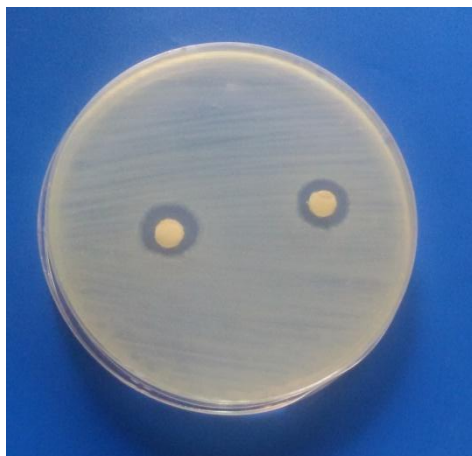


**(A) *Bacillus subtilis***

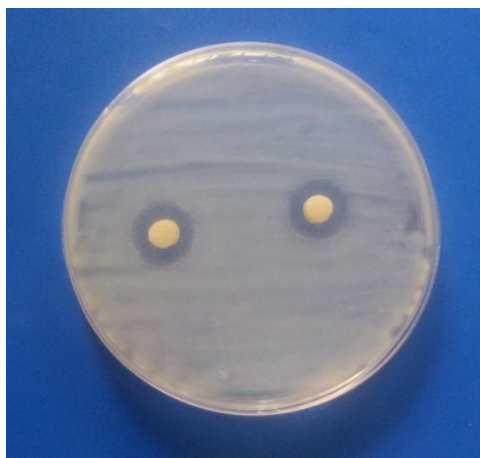


**(B) *Staphylococcus aureus***

**Fig 4.16: Anti-bacterial activity of Clotrimazol -Ag nanoparticle complexes (1mg/ml) against:**

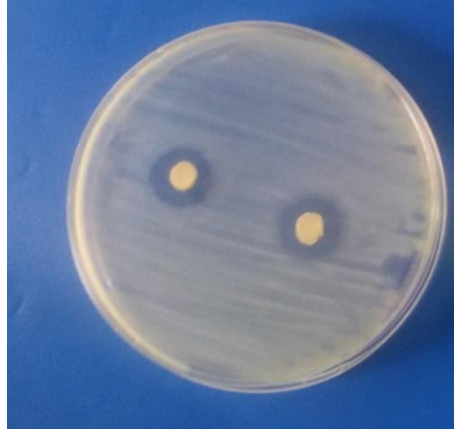


**(A) *Escherichia coli***

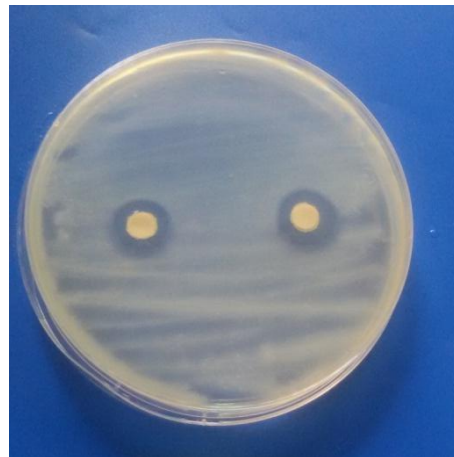


**(B) *Staphylococcus aureus***

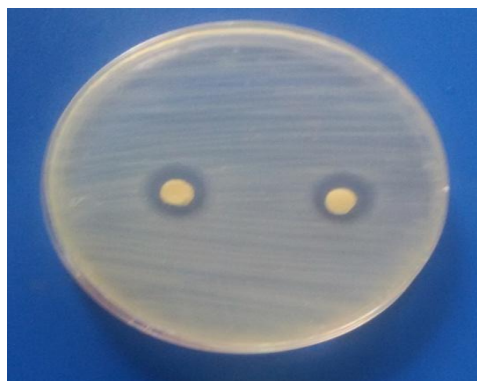
**Fig 4.17: Anti-bacterial activity of Clotrimazol -Ag nanoparticle complexes (0.5mg/ml) against**



**(A) *Staphylococcus aureus***

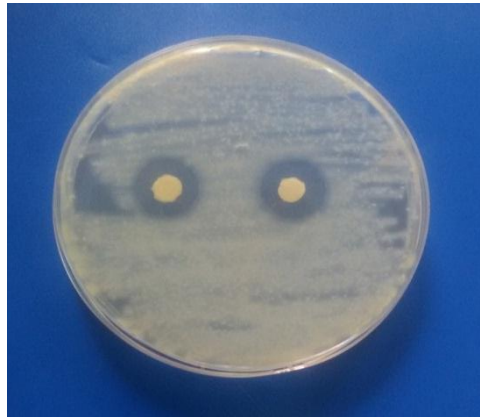


**(B) *Bacillus subtilis***

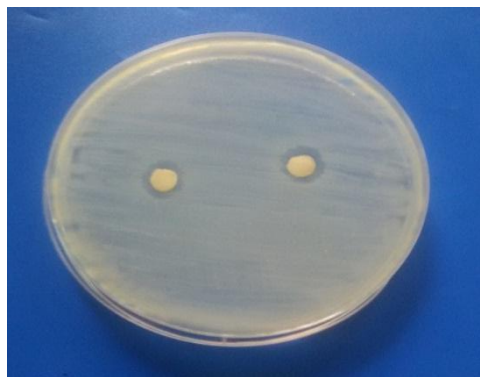


**(C) *Escherichia coli***

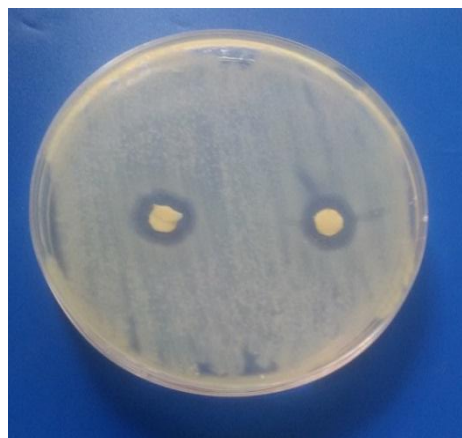
**Fig 4.18: Anti-bacterial activity of Tindazole -Ag nanoparticle complexes (1mg/ml) against *Bacillus subtilis***



**Fig 4.19: Anti-bacterial activity of Tindazole +Ag nanoparticle compound (2mg/ml) against**

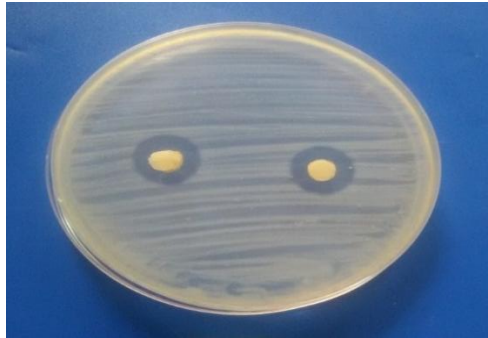


**(A) *Pseudomonas aeruginosa***



**(B) *Bacillus subtilis***

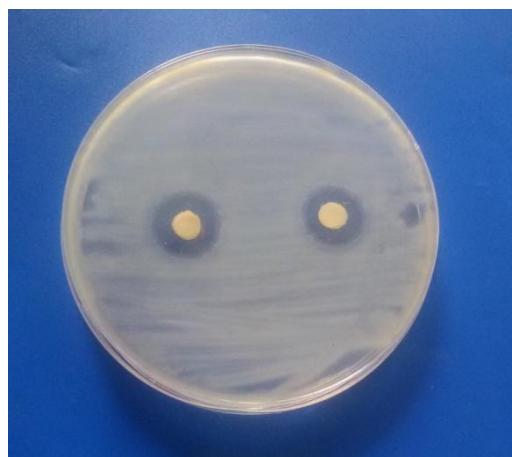
**Fig 4.20: Anti-bacterial activity of Tindazole -Ag nanoparticle complexes(0.5mg/ml) against**



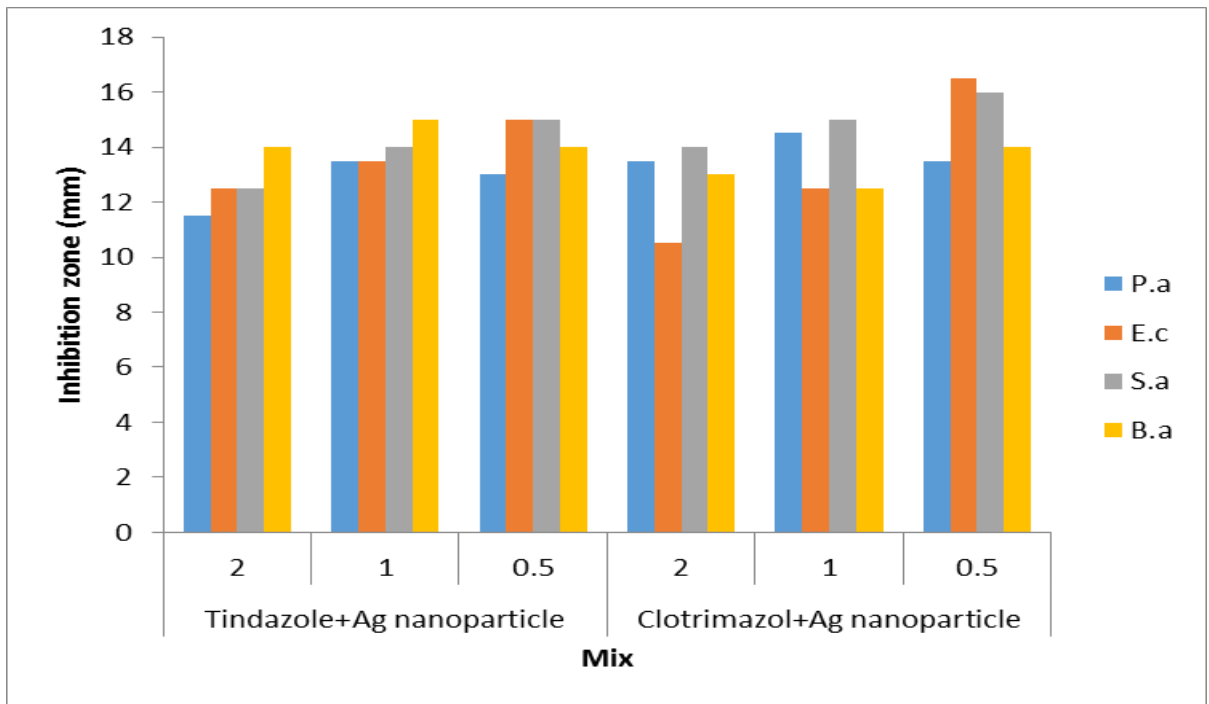
**(A) *Escherichia coli***



**(B) *Pseudomonas aeruginosa***



**(C) *Staphylococcus aureus***



**Fig 4.21: growth inhibition zone curve of (Tindiazole +AgNPs) and (Clotrimazole +AgNPs) complexes**

#### 4.4. Conclusions

In summary, silver nanoparticles with the size ranging between 50 to 55 nm were successfully synthesized by chemical reduction method using tri-sodium citrate as a reducing agent. The nanoparticles were characterized by UV/Vis and TEM. UV/Vis spectra show the characteristic plasmon absorption peak for the silver nanoparticles is 418 nm which confirms the presence of silver nanoparticles. Size and shape of the silver nanoparticles have been obtained from TEM micrograph. The shapes of silver nanoparticles are hexagonal with average size of 50 nm and a rod-like with average size of 55 nm approximately.

Silver nanoparticle solution was screened for its antibacterial activity against four standard bacterial species (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*). It has been found that the Ag NPs have considerable activity towards all tested bacterial species. Clotrimazole and tindiazole were combined with nanoparticles through overnight incubation and its inhibition zone also was found out, the results show a significant increase in antibacterial activity of antibiotics in the presence of silver nanoparticles.

The FT-IR data of clotrimazole showed an alteration in the wavenumber of some functional groups like C=C, NO<sub>2</sub>, C-N. Whereas, The FT-IR data of tindiazole showed an alteration in the wavenumber of some functional groups like C=C and C-N, as compared to control. It might be due to some alteration at the atomic level through complex formation.

A comparison of the relevant IR spectral bands of clotrimazole and tindiazole alone and with the silver nanoparticle indicated shifts in the frequencies as well as reductions in the band intensities, which proved the formation of charge transfer complexes. These shifts in the values were due



to changes in the electronic structures and molecular symmetries of reactants upon complex formation.

From the above results obtained, we can conclude that a silver nanoparticle plays a vital role in enhancing the antibacterial activity of clotrimazole and tindiazole. When Ag nanoparticles combined with clotrimazole and tindiazole also it was found to be effective when compared to the individual antibacterial activity of Ag nanoparticles as well as antibiotic.