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Biosynthesis of Silver Nanoparticles Using Plant Leaf Extracts of *Mentha spicata* and Study of Biological Activities

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Dedication

I would dedicate my effort to:

My angel in life, to the meaning of love and compassion who taught me the meaning of life and their existence is the reason for my success, who taught me to trust in Allah, believes in hard work and that so much could be done with little.

My parents

My strength, who gave me love, cooperation and support me.

My brothers and sisters

Amal

Summary

The present study described the rapid green synthesis of silver nanoparticles using three aqueous, ethanol and methanol leaf extracts of *Mentha spicata* as a reducing agent and stabilizing.

When a silver nitrate solution was exposed to leaf extract of *Mentha spicata*, led to reduce the Ag^{+} ions and produce change of colour that indicated the formation of silver nanoparticles.

Silver nanoparticles were characterized using UV-Visible spectroscopy which the peak showed between 450- 550 nm is the formation of plasmon of silver nanoparticles, spectrum of Fourier Transformation infera-red spectroscopy (FTIR) indicated that the presence of all the functional groups, Atomic Force Electronic Microscopic (AFM) showed the average diameter of silver nanoparticles which obtained 57.51, 76.40 and 91.87 nm, X-ray diffraction (XRD) technique revealed that the synthesis of silver nanoparticles.

Optimization studying for synthesis of silver nanoparticles such as concentration of silver nitrate, plant extract and effect of light (as inducer for biosynthesis of silver nanoparticles), previous studies showed that silver nitrate and plant extract for biosynthesis of silver nanoparticles were 1 mM of silver nitrate and 5% volume of extract.

The antibacterial activity of silver nanoparticles was studied against multidruge resistance bacteria causing urinary tract infection, at concentration 5mg/ml with the average inhibition zone diameter were 16.33-18.67, 19.5-21.83, 16.83-23.33, 16.83-22.33 and 16.83-19.83 for *Staphylococcus aureus*, *Escherichia coli*, *Enterococus faecalis*, *Proteus mirabilis* and *Acinetobacter baumannii*, respectively.

The antifungal activity of silver nanoparticles was studied against *Microsporum canis*, *Trichophyton mentagrophytes*; at 5mg/ml the percentage of inhibition were 100, 84.97-87.27, respectively.

Summary

Silver nanoparticles were evaluted as antioxidant and it had activity which is antioxidant at 250, 275 and 300 µg/ml which was compared with antioxidant of aqueous, ethanolic and methanolic leaf extract of *Mentha spicata* that had efficient to reduction absorbance free radical ABTs to half (IC_{50}) at 1000, 1250 and 1250 µg/ml, respectively.

Effect of silver nanoparticles on human red blood cells hemolysis was investigated, In general silver nanoparticles showed that the low RBCs hemolysis percent at 50 µg/ ml were (2.80%), (3.39%) and (4.84%) with aqueous, ethanolic and methanolic, respectively. Whereas at 60 µg/ ml was ratio hemolysis reed blood cells (4.50%), (4.24%) and (6.03%) with aqueous, ethanolic and methanolic, respectively.

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Abbreviations

AFM	Atomic force microscope
FTIR	Fourier transform infera red spectroscopy
XRD	X- ray diffraction
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
AgNPS	Silver nanoparticles
HIV	Human immunodeficiency virus
Aq.	Aqueous
Eth.	Ethanol
Meth.	Methanol
UTI	Urinary tract infection

INTRODUCTION

Introduction

Introduction

Nanotechnology has attracted a great interest in recent years due to its expected impact on many areas such as medicine, agricultures and industrial etc (Zhang *et al*., 2013).

Silver nanoparticles have antimicrobial due to their surface reactivity, biosynthesis of silver nanoparticles preferred on the chemistry and physical method in medical application because it has advantage ecofriendly and low toxic and cost while chemical and physical methods are toxic and not effective and high cost (Ahmed *et al.*, 2016).

Plant extracts contain phenolics, terpenoids, polysaccharides and flavones compound that contribute in reduction and stabilizing of silver nanoparticles (Kumar and Yadav, 2009).

Increasing multidrug resistance of pathogenic bacteria due to misuse of antibiotic and development in resistance of bacteria which related to resistance genes appeared, in addition to three main mechanisms for resistance (i) inactivation of the antibiotic by destruction or modification, (ii) prevention of access to the target, and (iii) alteration of the antibiotic target site. Alternative replacing antibiotic with silver nanoparticles which has antimicrobial activity against multidrug resistant of pathogenic bacteria, and it will be necessary to change the use of antibiotic so that these silver nanoparticles are administered only when all other treatments options have failed (Levin, 1993; Franci *et al.*, 2015).

Study it is cytotoxicity toward the red blood cell; it is a necessary component together with the check of their antibacterial activity (Golubeva *et al*., 2010).

In addition, activity of silver nanoparticles as antimicrobial has antioxidant due to capping agent for silver nanoparticles core (Mittal *et al.*, 2012).

Introduction

The current study aims to synthesis of silver nanoparticles by plant extract, study characteristic of silver nanoparticles and evaluation biological activity of silver nanoparticles.

The general steps of this study are:

- Synthesis of silver nanoparticles by aqeous, ethanolic and methanolic leaf extract of *Mentha spicata* as reducing and stabilizing agent.
- Study optimization of silver nanoparticles synthesis.
- Characterization of the silver nanoparticles using UV-Visible spectroscopy, Fourier Transform Infrared spectroscopy (FTIR), X-Ray Diffraction (XRD) and Atomic Force Microscope (AFM).
- Study evaluation of biological activity of silver nanoparticles as antibacterial, antifungal, antioxidant and it is effect on human red blood cells hemolysis.

CHAPTER ONE

1. Literature review

1.1 History of nanotechnology

Nanotechnology controlled manipulation of nanomaterials with at least one dimension less than 100 nm (Xia and Halas, 2005)

As mentioned that the word "nanotechnology" was first used in 1974 by Norio Taniguchi in a paper titled "on the Basic concept of Nanotechnology", by the 1980s, people were regularly using and publishing the word "nanotechnology", the late Richard smalley shared the 1996 Nobel prize in chemistry with Harry Kroto and Robert Curl, was a champion of the nanotech cause, in 1999 he told congress that "the impact of nanotechnology on the health, wealth, and lives of people will be at least the equivalent of the combined influences of microelectronics, medical imaging, computer aided engineering and manmade polymers ". Nanobiotechnology describes the applications of nanotechnology techniques for the development and improvement of biotechnological process and products (Gazit and Mitraki, 2013).

Nanobiotechnology is widely emerging field which involves interdisciplinary subject as biology, physics, chemistry, and medicine (Tiquia-Arashiro and Rodrigues, 2016).

The origin of the term "Nano" is Greek which means dwarf and denotes a measurement on the scale of one-billionth (10^{-9}) of a meter in size (Makeen *et al*., 2015).

Nanoparticles are clusters of atoms in size range of 1-100 nm, the physical and chemical properties of nanomaterial can become very different from those of the same material in larger bulk form (Rai *et al.*, 2009; Biswas and Dey, 2015).

Nanotechnology involves the synthesis of nanoparticles using the top-down and bottom-up methods. In top-down synthesis, nanoparticles are produced by size reduction from a suitable starting material size

reduction is achieved by various physical and chemical treatments as shown in figure (1). Top-down synthesis production methods introduce imperfections in the surface structure of the product and this is a major limitation because the surface chemistry and the other physical properties of nanoparticles are highly dependent on the surface structure. In bottom up synthesis, the nanoparticles are built from smaller entities, for example by joining atoms, molecules and smaller particles In bottom up synthesis, the nanostructured building blocks of the nanoparticles are formed first and then assembled to produce the final particle. The bottom-up synthesis mostly relies on chemical and biological methods of production. The probable mechanism of nanoparticle synthesis by bottom up approach is shown in figure (2) (Mittal *et al.*, 2013).

1.2 Nanoparticles

Nanoparticles are the building blocks of nanotechnology; they are defined as particles having more than one dimension measuring 100 nm or less. They behave differently from matter at the bulk scale (Kushwaha *et al.*, 2015).

Nanoparticles can be classified into organic nanoparticles which include carbon nanoparticles such as "fullerenes", and inorganic nanoparticles which include magnetic nanoparticles, Nobel metal nanoparticles such as "silver" and semiconductor nanoparticles such as "zinc oxide" (Panigrahi, 2013).

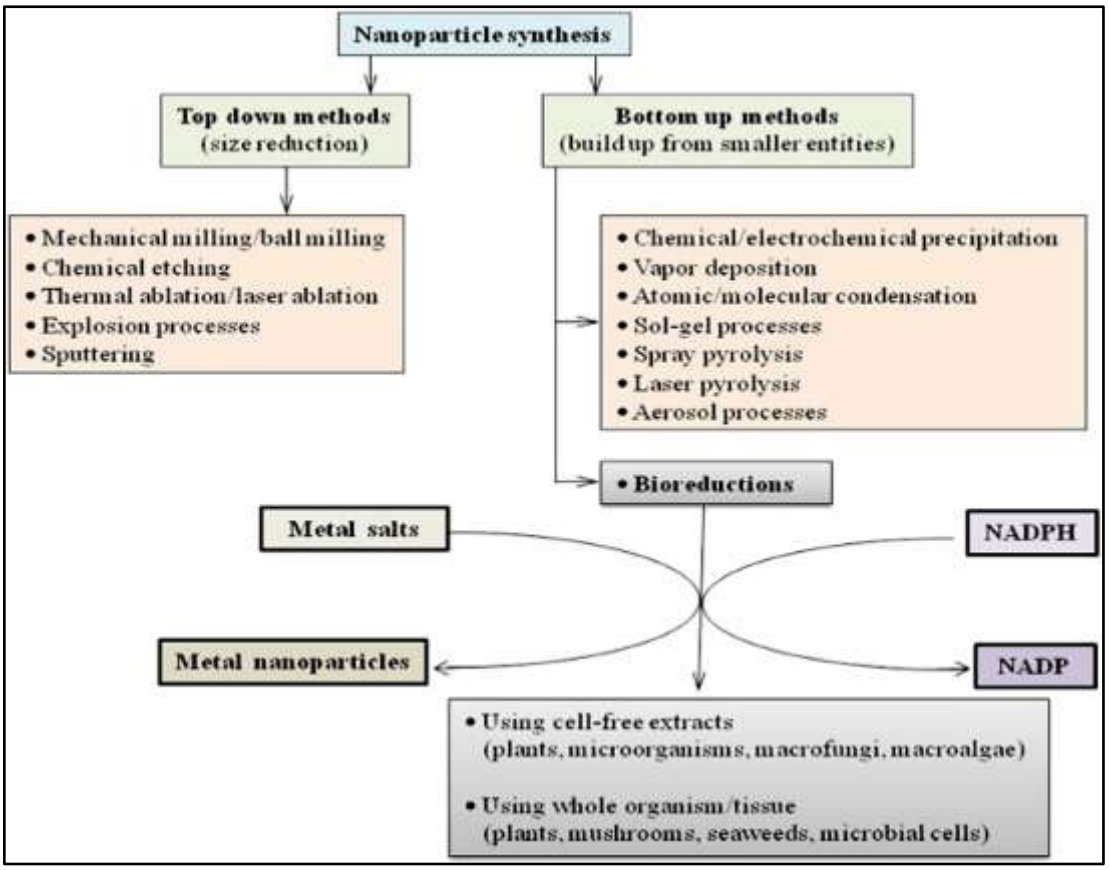


Figure (1): Various approaches for making nanoparticles and cofactor dependent bio reduction adapted from (Mittal *et al*., 2013)

Figure (2): Mechanisms of nanoparticle synthesis (M±metal ion) adapted from (Mittal *et al*., 2013)

1.3. Silver nanoparticles

Silver is a soft and hard, white, lustrous transition metal possessing high electrical and thermal conductivity. It has been known longer than

the recorded history due to its medical and therapeutic benefits before the realization that microbes as agent for infections. It is used in many forms as coins, vessels, solutions, foils, sutures, and collides as lotions, ointments, etc. It is the foremost therapeutic agent in medicine for infectious diseases and surgical infections. Silver benefits more than risk factors (Firdhouse and Lalitha, 2015).

Silver nanoparticles (AgNPs) are receiving great interest due to their application in diverse such as medicine, electronics, cosmetics, coatings, packaging, and biotechnology (Kushwaha *et al*., 2015).

1.4 Synthesis of silver nanoparticles

Generally, silver nanoparticles are prepared by a variety of methods:

1-Physical methods

2-Chemical methods3-biological methods

The disadvantages of the synthesis physical and chemical methods are more expensive, energy consuming and toxic to the environment and not suitable for biological applications, involving the use of toxic and perilous chemicals that are responsible for various biological risks. Furthermore, it cuts down on the necessity to use high temperature, pressure, energy and toxic chemicals (Ghaffari-Moghaddam *et al.*, 2014; Vanaraj *et al.*, 2017).

The advantages of biosynthesis methods are cost effective and ecofriendly; capping and stabilizing are found in extract of plant or microbial cultures are therefore nontoxic (Zuas *et al*., 2014).

The synthesis of silver nanoparticles by biological methods (biosynthesis) can be synthesized by microbial or extract of plant.

1.4.1 Synthesis of silver nanoparticles by microorganisms

Recently many studies were conducted to explore the synthesis of silver nanoparticles using microorganisms as a potential biosource,

synthesis of silver nanoparticles which uses microorganisms or supernatant of microbial culture at rates much slower than the rates in which plants can biosynthesise silver nanoparticles. The time required for the complete reduction of silver ions is known to range from 24 hours to 120 hours in the case of microorganisms, while the reaction completion time is much less in the case of plants, ranging from a few hours to a maximum of 48hours (Sabri *et al*., 2016).

1.4.1.1 Synthesis of silver nanoparticles by bacteria

Synthesis of nanoparticles can be extracellular or intracellular depending upon bacterial species. There is a limit of nanoparticles accumulation up to which bacteria can survive; after that, nanoparticles accumulation can be toxic for the microbes (Duhan and Gahlawat, 2014).

The first evidence of silver nanoparticles production from bacteria *Pseudomonas stutzeri* AG259 strain was isolated from silver mine, when placed in silver nitrate solution; it produces silver nanoparticles in its periplasmic space which are pyramidal and hexagonal shape and size up to 200 nm (Klaus *et al*., 1999).

Many previous studies indicate that silver nanoparticles synthesized by bioreduction of silver nitrate with a culture supernatant of *Bacillus licheniformis* (Kalishwaralal *et al*., 2008), furthermore the synthesis of extracellular silver nanoparticles was used culture supernatant of *Pseudomonas aeruginosa* (Kumar and Mamidyala, 2011).

There are some microorganisms that can survive metal ion concentration and can also grow under those conditions, and this phenomenon is due to their resistance to that metal. The mechanisms involved in the resistance are efflux system, alteration of solubility and toxicity via reduction or oxidation, biosorption, bioaccumulation, extracellular complex formation or precipitation of metals, and lack of specific metal transport systems, though microorganisms can grow at

lower concentration but exposure to higher concentrations of metal ions can induce toxicity (Husseiny *et al*., 2007).

The most widely accepted mechanism of silver biosynthesis is the presence of the nitrate reductase enzyme, which converts nitrate into nitrite, invitro synthesis of silver nanoparticles using bacteria, the presence of alpha-nicontinamide adenine dinucleotide phosphate reduced from (NADPH) dependent nitrate reductase would remove the downstream processing step that is required in other cases as show in figure (3) (Kalimuthu *et al.*, 2008).

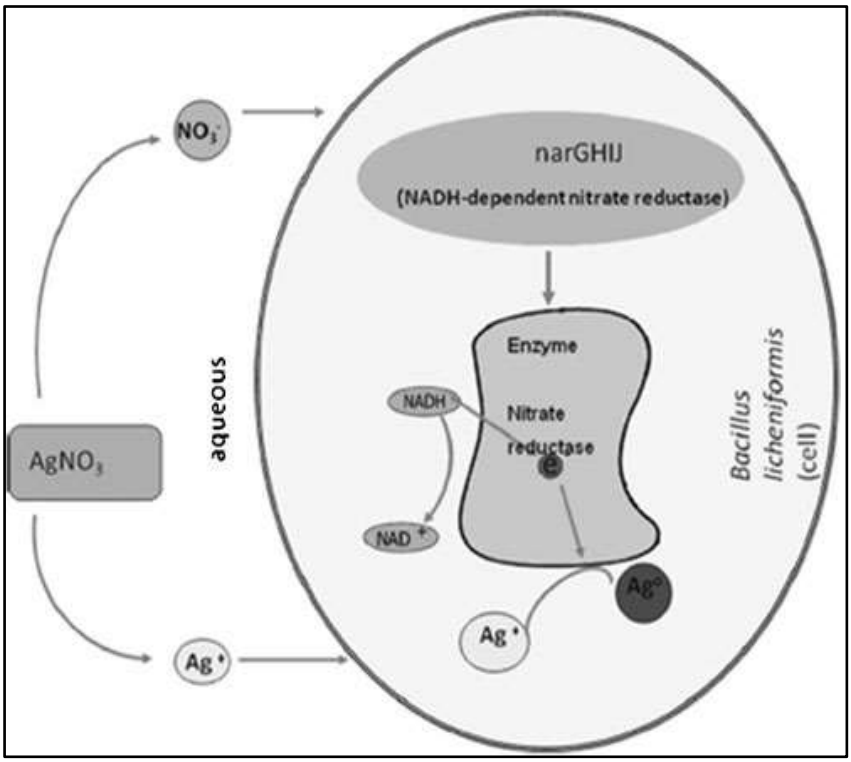


Figure (3): Possible mechanism for silver nanoparticle synthesis in *B.licheniformis* adapted from (Kalimuthu *et al.*, 2008).

1.4.1.2 Synthesis of silver nanoparticles by fungi

Several fungi have been widely used for the biosynthesis of silver nanoparticles. It can be compared to bacteria, and fungi could be a source for large amount production of nanoparticles. Since fungi are known to secrete much higher amounts of proteins, there might be significantly

higher productivity of nanoparticles in biosynthetic approach (Mohanpuria *et al*., 2008).

Mechanisms of synthesis of silver nanoparticles by reducing silver nitrate to silver nanoparticles by nitrate reductase enzyme, nitrate dependent reductase presents perhaps, the most prevalent enzyme observed in bioreduction processes in microbes such as *Aspergillus flavus* which is capable of synthesis of silver nanoparticle on the surface of the cell wall when incubated with silver nitrate solution. Expectedly, nanoparticles produced in an extracellular manner were stabilized by the protein and reducing agents secreted by the fungus or NADH-Dependent Reductase-Mediated Reduction such as *Fusarium oxysporum* protein assays has indicated that NADH-dependent reductase is the main enzyme responsible for nanoparticle biosynthesis. This enzyme is first conjugated with an electron donor (quinine); the resultant conjugate then reduces the metal ion and changes it to elemental form, this reductase gains electrons from NADH and oxidizes it to NAD^+, the enzyme is then oxidized by the simultaneous reduction of metal ions as shown in figure (4) (Duran *et al*., 2005; Devi and Joshi, 2015).

The silver ions were firstly adsorbed on the mycelial surface through the interactions with the chemical functional groups such as carboxylate anion and carboxyl and peptide bonds of proteins; then the reduction process was held by reducing sugar from the saccharides on the mycelia; it was believed that protein acting as a capping agent was responsible for the stabilization of silver nanoparticles (Vigneshwaran *et al*., 2006).

Several researchers have attempted to use fungi as reduction and stabilization for synthesis of silver nanoparticles as mentioned by Bhainsa and Dsouza (2006) using filamentous fungus *Aspergillus fumigates*, Prabakaran *et al*. (2016) used entomopathogenic fungus *Beauveria*

bassiana, and Nadhim Owaid *et al*. (2017) used oyster mushroom's extracts to synthesis of silver nanoparticles.

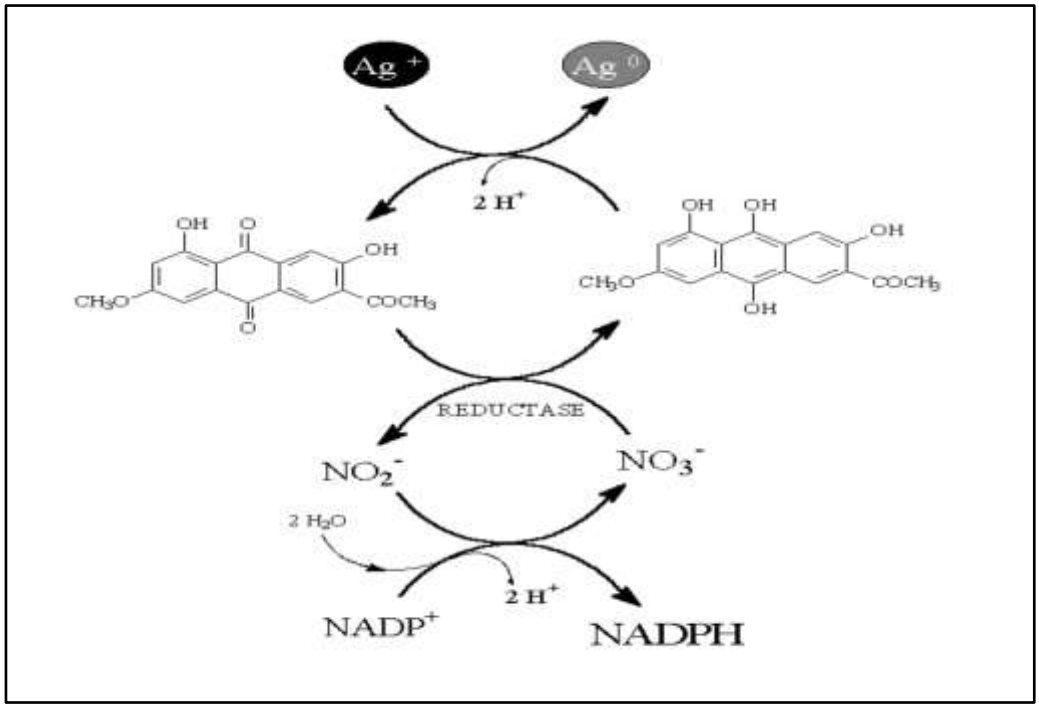


Figure (4): Hypothetical mechanisms of silver nanoparticles biosynthesis adapted from (Duran *et al*., 2005)

1.4.2 Synthesis of silver nanoparticles by plants

The plant mediated synthesis of nanoparticles is more advantageous than other biological process as there is no troublesome of preserving and maintaining the cell culture. Plant mediated nanoparticles synthesis is easy, one step synthesis method with no chances of mutation as in microorganisms. Extraction and separation can be easily scaled up for the large-scale synthesis of nanoparticles (Veerasamy *et al*., 2011).

Production of microorganisms is often more expensive than the production of plant extracts (Mittal *et al*., 2013).

Green synthesis of nanoparticles using phytocompounds as bio-reductants is attaining a greater impetus, a variety of plant materials, such

as leaf extracts, fruit, bark, fruit peels, root and callus (Rai and Yadav, 2013).

Flavonoid, terpenoid and alkaloids compounds present in the extract were claimed to be responsible for reduction and stabilization of nanoparticles as shown in figure (5) (Huang *et al*., 2007; Dubey *et al*., 2009).

The presence of some phytochemical compounds such as flavonoids and phenolic in the extract of *M.pendan* may be responsible for the activity (Zuas *et al*., 2014).

Rapid green synthesis of silver nanoparticles using fresh leaves of *Cymbopogan Citratus* and silver nanoparticles were formed within 8-10 minutes by microwave irradiation (Masurkar *et al*., 2011).

Plant-mediated synthesis of silver nanoparticles using the extract of *A.sessilis* by sonication method advocates green nanotechnology (Lalitha, 2015).

Sunlight induced rapid biosynthesis of silver nanoparticles using an *Andrachnea chordifolia* ethanol extract (Zarchi *et al*., 2011).

Photo induced reduction methods are gaining wide attention due to the following advantages: (1) controlled reduction of metal ions can be carried out without using excess of reducing agent; (2) radiation is absorbed regardless of the presence of light absorbing solutes and products and (3) photo assisted methods are more competitive and cost effective (Dong *et al*., 2004).

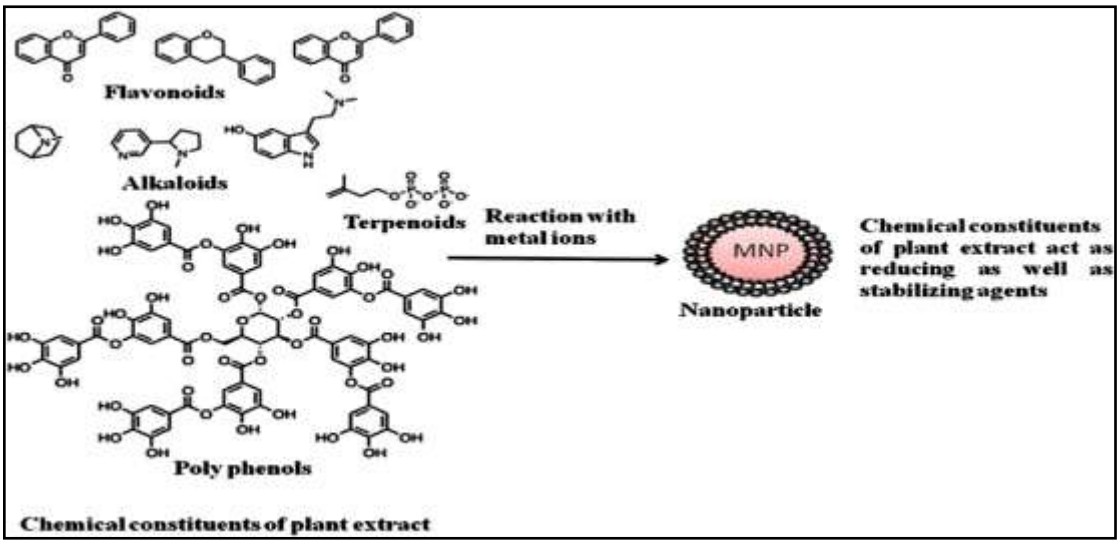


Figure (5): Possible chemical constituents of plant extract responsible for the bioreduction of metal ions adapted from (Huang *et al.*, 2007)

1.5 Mentha spicata L. synonym: M. viridis (spearmint)

Kingdom: plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Lamiaceae (Labiatae)

Genus: *Mentha*

Species: *spicata*

Classification is cited by (Gaurav, 2016).

The genus *Mentha* (also known as mints) is a member of the Lamiaceae (Labiatae) family consisting of about 25–30 species, originates from Eastern Asia and there are two major forms, *Mentha piperita* (peppermint) and *Mentha spicata* (spearmint) (Ali *et al*., 2002).

The historical use of *Mentha* is not different from its use in modern herbal medicine. *Mentha* can be used for common cold, cough, sinusitis, fever, bronchitis, nausea, vomiting, indigestion, antimicrobial, antioxidant, intestinal colic and loss of appetite. It is also used for

flavoring chewing gums, toothpaste, confectionery and pharmaceutical preparations (Saleem *et al*., 2000; Starburck, 2001).

Mints are regarded as one of the most important spices throughout the world. The essential oils of mints are widely used as flavorings in the food, cosmetic, and pharmaceutical industries; the chemical composition of the essential oils in spearmint has been studied by different researchers. Carvone is the major component in all cases and is the character-impact component in spearmint, followed by limonene and 1,8-cineole (Barton *et al*., 1992; Marongiu *et al*., 2001; Pino *et al*., 2001).

Mentha is known to produce a wide range of natural terpenoids named menthol ($C_{10}H_{20}O$) found in the essential oils of the mints family (*Mentha* spp.) as mentioned by Mickiene *et al*. (2011).

1.6 Urinary Tract Infection (UTI)

Urinary tract infection is an extremely common condition that occurs in both males and females of all ages. The prevalence and incidence of urinary tract infection is higher in women than in men, which is likely the result of several clinical factors including anatomic differences, hormonal effects, and behavior patterns (Griebling, 2007).

Urinary tract infections may involve just the lower tract or both the lower and upper tracts, it is generally accepted that 10^5 or more CFU/ml of urine is significant bacteriuria, thought the patients may be symptomatic or asymptomatic. *E.coli* causes 80- 90% of acute uncomplicated bacterial lower tract infections, *E.coli* is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women (Foster, 2008; Neal, 2008).

Urinary tract infection is caused by pathogenic invasion of the urinary tract, with resultant of an inflammation, including acute or chronic infections. The clinical signs of UTI depend on the portion of the urinary tract involved, pathogen, the intensity of pathogenicity, immune

response of patient. Signs and symptoms may include fever, chills, dysuria, urinary urgency, frequency, and cloudy or malodorous urine. Bacteriuria refers to bacteria that presents in the urine, but is not equivalent to urinary tract infection. UTI is invasion of bacteria that results the inflammatory response and the associated signs and symptoms. Pyuria refers to a presence of white blood cells in the urine that related of inflammation in response to bacterial infection but without asymptomatic (Valiquette, 2001; Dielubanza and Schaeffer, 2011; Salvatore *et al*., 2011).

1.7 Application of silver nanoparticles:

Many previous studies that metallic nanoparticles show different applications as industrial and medical applications were nanoparticles synthesized by the various methods (Mittal *et al*., 2013).

Nanoparticles have been used as therapeutic agent, imaging diagnosis and delivery vehicles for drugs and genes due to the size range of 2-100 nm of nanoparticles can interact with biological systems as the molecular level and allow targeted delivery and passage through biological barriers (Kim and Hyeon, 2013).

Silver nanoparticles have antimicrobial properties and also have shown to prevent human immunodeficiency virus (HIV) bending to host cells. In addition silver has been used in water and air filtration to eliminate microorganisms (Sharma *et al*., 2009).

Nanoparticles are commercially used in textile industries, silver nanoparticles were incorporated into fiber or coated on fiber or cotton, the characteristic of the treated fibers with silver nanoparticles give a dual size roughness on the textile which confer its superhydrophobic surfaces and coating fiber gave insulating cotton textiles conductivity, had high antibacterial activity against the gram negative bacteria *Escherichia coli*

theses multifunctional textiles might find applications in biomedical electronic devices (Xue *et al*., 2012).

Silver nanoparticles are widely used in consumer product namely soaps, food, plastics, pastes and textile due to their fungicidal and bactericidal activates (Guo *et al*., 2016).

Attention is given to biosynthetic routes exploiting the bio polymeric matrix and to the methods allowing preparing magnetically controllable antimicrobial polymers for targeting to an active place (Dallas *et al*., 2011).

1.7.1 Mechanism of action of silver nanoparticles:

Silver nanoparticles are an important broad spectrum antimicrobial agent and have been extensively applied in biomedical fields (He *et al*., 2017).

The mechanism of the bactericidal effect of silver nanoparticles may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell, antibacterial properties of silver particles are size dependent with smaller particles exhibiting a greater effect due to surface area of the particles, for instance smaller silver nanoparticles having the large surface area available for interaction would give more bactericidal effect than the larger silver nanoparticles (Sahayaraj and Rajesh, 2011; Fadeel, 2014; Anbukkarasi *et al*., 2017).

Silver nanoparticles internalized bacteria and the cell energy is reduced due to the decline in the adenosine triphosphate levels and the essential energy molecule and destabilization of the outer membrane, there is a strong trend that considers two mechanisms underlying the interaction of nanoparticles with bacteria, to be mainly concerned (i) excessive reactive oxygen species generation mostly hydroxyl radicals and singlet oxygen and (ii) nanoparticles precipitation on the bacterial exterior or nanoparticles gather in the cytoplasmic area or in the

periplasm space thus disrupt the cellular activities resulting in membranes disturbance and disorder as shown in figure (6) (Sirelkhatim *et al*., 2015).

Silver nanoparticles react with sulfur containing amino acids inside or outside the cell membrane leading to the inhibition of enzyme functions, also interact with phosphorus moieties in DNA resulting in inactivation of DNA replication, due to high affinity of silver towards sulfur and phosphorus as show in figure (7) (Prabhu and Poulose, 2012; Sulaiman *et al*., 2013; Zhang *et al*., 2013).

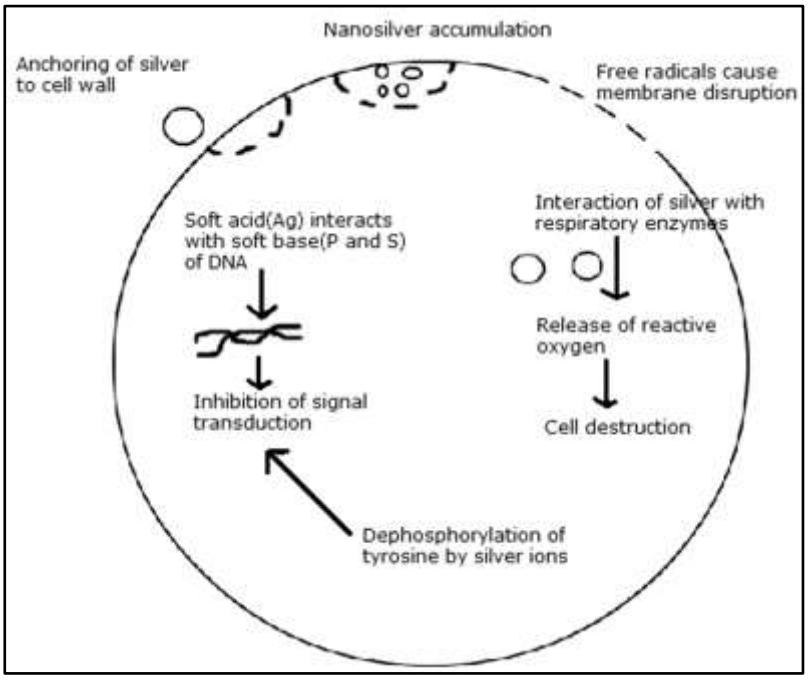


Figure (6): Various modes of action of silver nanoparticles on bacteria adapted from (Sirelkhatim *et al*., 2015)

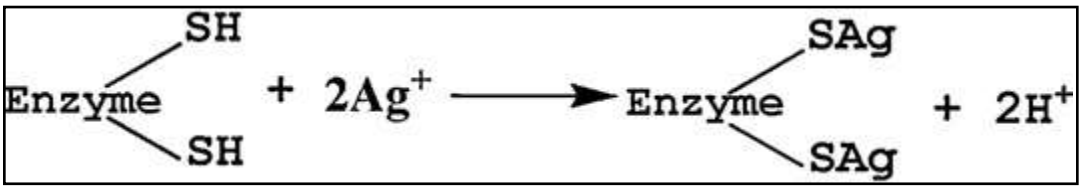


Figure (7): The antibacterial mechanism of silver nanoparticles adapted from (Prabhu and Poulose, 2012)

1.7.2 Antifungal activity of silver nanoparticles

Dermatophytoses which are skin diseases caused by a group of fungi called dermatophytes, the genus was responsible that are *Epidermophyton*, *Trichophyton* and *Microsporum* (Ajello, 1974).

Several studies found that antifungal activity of silver nanoparticles against pathogenic fungi, most of the studies were on the effect of silver nanoparticles on *candida* species (Chwalibog *et al*., 2010; Monteiro *et al*., 2011; Nasrollahi *et al*., 2011).

However few studies have evaluate silver nanoparticles activity on the dermatophytes, the evaluation antifungal activity of biosynthesized silver nanoparticles in footwear material against dermatophytes and other fungi (Falkiewicz-Dulik and Macura, 2008), also reported the activity of silver nanoparticles against *Trichophyton rubrum* (Pereira *et al*., 2014), in addition, study antifungal activity of silver nanoparticles against *Trichophyton rubrum* using disc diffusion method (Marcato *et al*., 2012). While antifungal activity of silver nanoparticles was synthesized using chemical method against clinical isolate of *Trichophyton mentagrophytes* (Kim *et al*., 2008).

1.7.3 Antioxidant activity of silver nanoparticles

Antioxidants are our first line of defense against free radical damage and are critical for maintaining optimum health and wellbeing, antioxidant activity of a molecule is attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts and decomposition of peroxides (Gulcin *et al*., 2003; Rakesh *et al*., 2010).

Free radicals can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital (Lobo *et al*., 2010).

Dauthal and Mukhopadhyay (2013) reported that the antioxidant potential of silver nanoparticles were synthesized using apricot fruit extract.

The *invitro* antioxidant properties of the biosynthesized silver nanoparticles have been evaluated and these nanoparticles were found to have higher antioxidant capacity compared to the seed extract and thus can be used as potential radical scavenger against deleterious damages caused by the free radicals, the synthesized nanoparticles have antioxidant activity due to capping can be used against deleterious of free radicals (Kandakumar *et al*., 2014).

The reactivity of nanoparticles with reactive oxygen species and reactive nitrogen species depends on:

(i) The catalytic potency of nanoparticles surface

(ii) Adsorption of molecules onto their surface.

(iii) Partial dissolution of nanoparticles and resulting release of metallic ions (Tournebize *et al*., 2013).

1.8 Toxicity of silver nanoparticles

Toxicity is not satisfactory recognized. However; some results reported toxicity only in the cases of exposure at high concentrations. It may suggest that a silver nanoparticle bears no risk in considered applications (Chladek *et al*., 2016).

Silver nanoparticles of 20 nm were more toxic than larger nanoparticles. In addition, the potency of silver in the form of nanoparticles to induce cell damage compared to silver ions is cell type and size dependent (Park *et al*., 2011).

Poly (diallyldimethylammonium) coated silver nanoparticles were found to be the more toxic than uncoated or colloidal silver nanoparticles which were found less toxic to both macrophage and lung epithelial cells (Suresh *et al*., 2012).

Silver nanoparticles were tested in Swiss mice and was non toxic, orally administered nanoparticles silver was not toxic to guinea pigs at acute doses of 5000 mg/kg of bw/day and hematological analysis showed no differences in any of the parameter examined (Maneewattanapinyo *et al.*, 2011).

The argyria is the most frequent adverse outcome from exposure to silver nanoparticles (Chang *et al*., 2006).

Orally administered silver was absorbed at a range of 0.4-18% in mammals with a human value of 18% and distributed to all of the organs (Hadrup and Lam, 2014).

The exacerbated risks arising from long term retention of silver nanoparticles in the brain despite its clearance from the most organs such a sliver and the broad range of their applications have drawn a lot of our attention to the importance of resorting to natural products that lessen their neurotoxic effects (Ahmed and Hussein, 2017).

Silver nanoparticles evoked their neurotoxicity through three main pathways, oxidative stress, mechanical damage and increase cytoplasmic Ca^{+2} (Fedorovich *et al*., 2010).

Mechanisms of toxicity are still poorly understood although it seems clear that in some cases nano scale specific properties may cause bio uptake and toxicity over and above that caused by the dissolved silver ion (Fabrega *et al*., 2011).

Silver nanoparticles based in medicine will be introduced into the vascular system via injection or orally administration, direct interaction between silver nanoparticles and RBCs may occur frequently (Chen *et al*., 2015).

The earlier report on silver nanoparticles has clearly demonstrated that small particles have higher hemolytic activity compared to large particles, in contract that in silica nanoparticles increasing hemolytic

activity increased with large particles (Yu *et al*., 2011; Zhao *et al*., 2011; Lubick, 2008).

As shown in a previous study that adsorption and take of silver nanporticles by RBCs were mentioned through dark field imaging, all size of nanoparticles were attached to RBCs surface or internalized into cytoplasm as show in figure (8) (Chen *et al*., 2015).

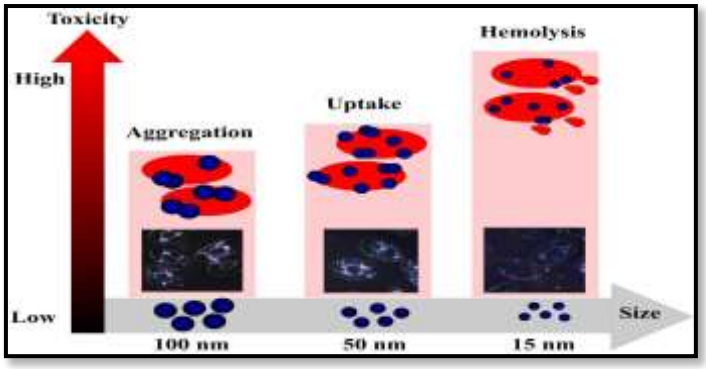


Figure (8): Size-dependent adsorption, uptake and hemolytic activity adapted from (Chen *et al*., 2015).

CHAPTER TWO

2. Materials and Methods

2.1 Materials

Table (2-1.1): Equipments and instruments used in this study

Apparatuses/instruments	Company (Origin)
AFM	Advanced Angstrom Inc/ USA
Autoclave	LabTech/ Korea
Cooling Centrifuge	Beckman/ USA
Digital Sensitive Balance	Sartorius/ Germany
FTIR	Shimadzu (84005) / Japan
GC-Gas Chromatography–Mass Spectrophotometer	Shimadzu / Japan
Hot plate with magnetic stirrer	LabTech/Korea
Incubator	Memmert/ Germany
Micro pipette	Humapette/ Germany
Microscope	Olympus/ Japan
Oven	Binder/ China
pH Meter	Memmert/ Germany
Shaker incubator	LabTech/ Korea
Ultrasonic processor	Daigger/ USA
UV-Vis spectroscopy	Cecil Aquarius CE 7200/ England
Vortex	ROMA / Italy
Water bath	Julabo/ Germany
Water Distillator	LabTech/ Korea
XRD	Bruker 2010/D2Phaser (Germany)

Table (2.1.2): Chemicals and biological materials

Material	Company/ Origin
2,2-azinobis-3 ethylbenzothiazoline 6-sulfonic acid (ABTS)	Himedia/ India
Antibiotic disk	Bioanalyse- Turkey
Api 20 , Api staph	Biomerieux- France
Blood agar	Himedia- India
DMSO	Loba chemie – India
Eosin methylene blue agar	Himedia- India
Ethanol	Scharlau-European Union
Glacial acetic acid(CH3COOH)	GCC-UK
Kit gram stain	Albasheer scientific bureau
MacConkey agar	Himedia- India
Mannitol salt agar	
Methanol	Scharlau-European Union
Muller Hinton agar	Himedia- India
Nutrient broth	
Potassium hydroxide (KOH)	Riedel- dehaen/Germany
Potassium Persulfat	BDH/England
Sabouraud dextrose agar	Himedia- India
Sliver nitrate (AgNO3)	EvonIK
Sodium Chloride (NaCl)	Thomas Baker –India
Tetramethyl-p-phenylene diamine dihydrochloride	BDH

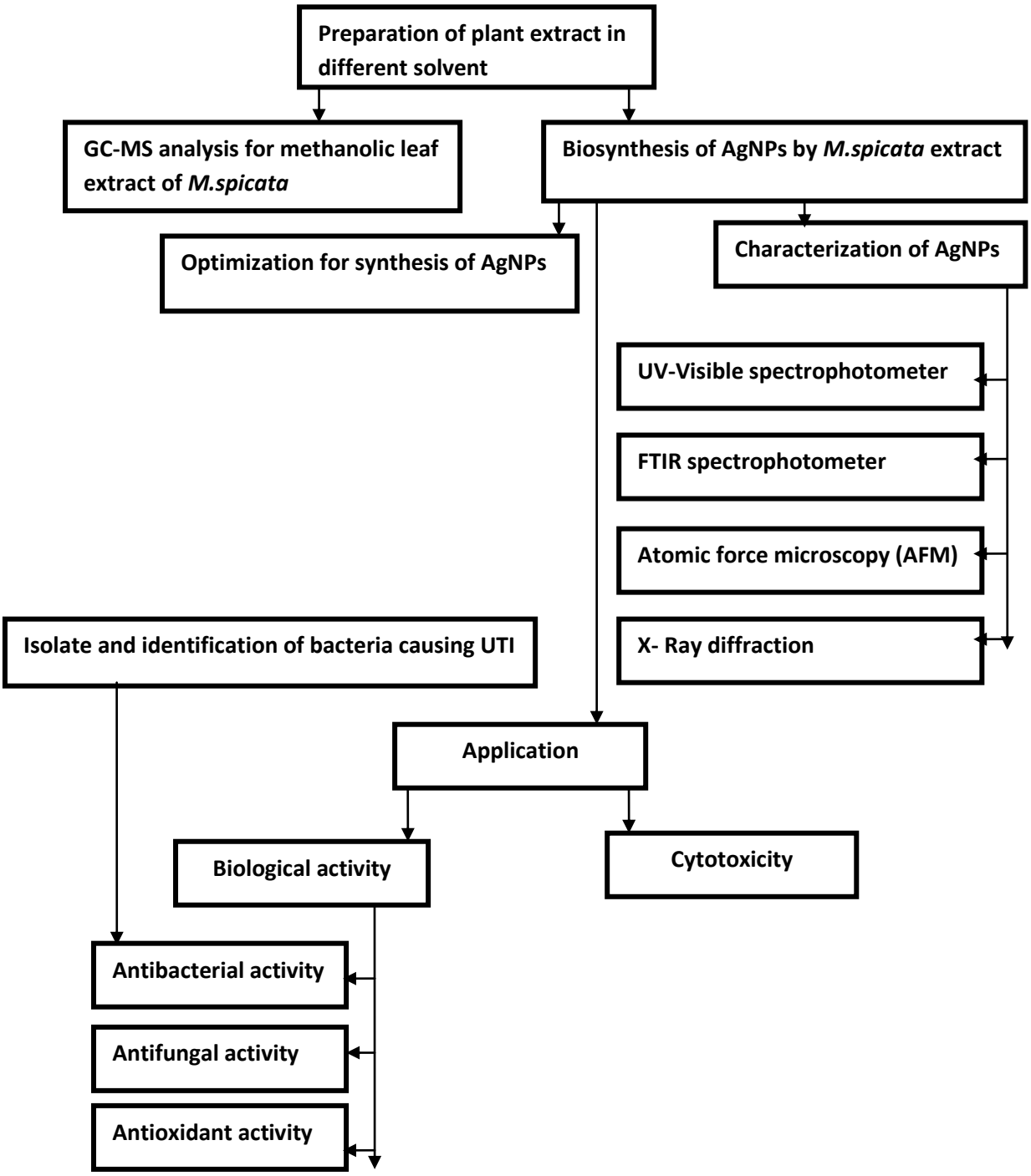


Diagram (1): The main steps of the study

2.2 Methods

2.2.1 Preparation of the plant extract

Fresh leaves of *M. spicata* were collected from region of kerbala, and washed many times in distilled water and then dried and grinded to form powder. Then plant extract was prepared by suspending 10 g of the powder in 250 ml solvents (water, ethanol and methanol) using ultrasonication for 1hours, then suspension was shaking for 1 h at last filtered by san rings filter paper (cold extract) (Kajani *et al*., 2014). 10 g of the powder in 250 ml water were boiled for 10 minutes (hot extract) and then complete the same steps mentioned (Awwad *et al*., 2014).

2.2.2 Gas chromatography/ Mass Spectrometry (GC/MS)

The component identification was achieved by the GC-MS analysis using shimadzu GC-MS 2010, in Basra University. Helium was used as carrier gas and 8 µL of *M. spicata* extract was injected into GC-MS using a micro syringe, shown in table (1) condition of work apparatuses. After getting chromatogram of GC-MS for organic compound, processing result in program correspondence isolate peak to identify of the organic compounds in the extracts which was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library; compounds were identified by comparing their spectra to those of the NIST08.LIB mass spectral libraries.

Table (1): Conditions of work apparatuses GC-MS

apparatuses type	Shimadzu GC-MS 2010 , Gas Chromatography- Mass Spectrophotometry
Column oven temperature	40.00 ºC
Column separation chromatography	Rtx-5MS capillary column (crossbond 5% diphenyl-95% dimethylpolysiloxane) , 30

	m(L) ×0.32 (i.d.) with a 0.25 µm film thickness
Gas	Helium
Injection mode	Split at ratio 1:30
Injection temperature	280.0 ºC
Ionsoure temperature	200 °C
Mass spectrophotometry	Electron Impact Ionization (EI) ; recorded in intervals from 20-500 m/z
Pressure	49.5 kpa
Sample injection volume	8 µL

2.2.3 Synthesis of silver nanoparticles

Silver nitrate solution was added to plant extract, reduction of silver nitrate to silver nanoparticles, and colour change from yellow to red-brown in aqueous solution due to the surface Plasmon resonance phenomenon give a peak at450-550 nm in UV-Visible spectrophotometer.

2.2.4 Study optimization for synthesis of silver nanoparticles

Silver nitrate solution (1mM, 2mM, 3mM and 4mM) was added to 5%, 10%, 15% and 20% of leaf *M. spicata* extract (with different solvent leaf extract of *M. spicata*), in state light bright (shadow), sun light direct induced synthesis of silver nanoparticles, synthesis of silver nanoparticles by hot leaf extract of *M. spicata* in dark.

2.2.5 Characterization of silver nanoparticles

The initial characterizations of silver nanoparticles were monitored by CECIL7200 UV–visible spectrophotometer the wavelength range between 200 and 700 nm. *M. spicata* leaf aqueous extract was utilized as the blank. Centrifuged at 12000 rpm for 30 minutes and wash three times with de-ionized water, the pellet of silver nanoparticles dried in oven at

40 ºC, grinding and kept in dark ambient. Other characterization of silver nanoparticles powder was obtained analyzed with X-ray diffraction (XRD) using a Bruker 2010/D2Phaser Using Cu Kα1 radiation (λ= 1.540562 Aº) at 30 mA current and 40 kV voltages. Fourier Transform Infrared (FTIR) Spectra was recorded by using a spectrophotometer (Shimadzu/84005), the powdered sample was mixed with KBr. The scans recorded were in the range between 4000- 400 cm^{-1}. And the surface morphology of sample was observed by Atomic Force Microscope (AFM) model, AA300, Advanced Angstrom Inc- USA.

2.2.6 Isolation and identification of bacteria

Preparation of culture media for isolation and diagnosis

Blood Agar base:

It was prepared according to the instructions of the supplied company by dissolving 40 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 ºC then human blood was added to the medium mixed gently after that poured into petri dishes. It is used for primary isolation and to detect the ability of bacteria to hemolysis red blood cells (RBCs).

MacConkey Agar:

It was prepared according to the instructions of the supplied company by dissolving 50 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes. It is used to isolate gram negative bacteria to differentiate lactose fermenters from none lactose fermenters.

Mannitol salt Agar:

It was prepared according to the instructions of the supplied company by dissolving 111 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes. It is used for selective and differential medium for *S.aureus*.

Eosin methylene blue (EMB) Agar:

It was prepared according to the instructions of the supplied company by dissolving 36 g of the medium in 1 liter of distilled water. It is used to differentiate *E. coli* from other Enterobacteria.

Preparation of reagent and solution used in isolate and diagnosis of bacteria cause urinary tract infection

1. Catalase Reagent

The reagent composed of 3% hydrogen peroxide H_2O_2 (Prescott *et al*., 2002).

2. Oxidase Reagent

It is freshly prepared from adding 0.1 g of tetramethyl – phenl – diaminedihydro chloride (BDH) in 0.9 ml of distilled water, then completed to 10 ml in clean, dark and sterile container (Prescott *et al*., 2002).

3. Gram stain kit

It consists of crystal violet stain, iodide solution, ethanol, and safranine stain.

4. Antibiotic discs

No.	Antibiotic	Symbol	Concentration (mcg)
1	Amikacin	AK	30
2	Amoxicillin-Clavulanic Acid	AMC	20/10
3	Ampicillin	AM	10
4	Azithromycin	AZM	15
5	Aztronam	AT	30
6	Ceftazidime	CAZ	30
7	Ceftriaxone	CTR	30
8	Chloramphenicol	C	30
9	Clindamycin	CD	2

10	Erythromycin	E	15
11	Gentamycin	GEN	10
12	Imipenem	IPM	10
13	Levofloxacin	LEV	5
14	Nalidixic acid	NA	30
15	Netilmicin	NET	30
16	Ofloxacin	OFX	5
17	Pencillin	P	10 unit
18	Rifampin	RA	5
19	Tobramycin	TOB	30

5. Api Systems (bioMerieux)

Api 20E and Api staph are identification system for Enteriobacteriaceae and staphylococcus respectively. Each system contains 20 standardized and miniaturized biochemical tests and database. These Api systems include a strip consisting of 20 microtubes containing dehydrated substrates. These tubes are inoculated with a bacterial suspension that re-constitutes the media.

During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index.

6. Standard Macfarland tube No. 0.5

Macfarland solution was prepared as follows:

A - 1.175 % $BaCl_2$ -$2H_2O$ in distilled water.

B - 1% H_2SO_4 (1ml of H_2SO_4 and the volume completed by adding 99 ml of distilled water).

Then 0.5 ml of $BaCl_2$ -$2H_2O$ a solution adding to 99.5 ml of H_2SO_4 solution, this mixture was shaken well and placed in a screw capped test tube and kept in dark at 4°C (Macfaddin, 2000).

7. Normal Saline Solution

0.85 g of NaCl was dissolved in amount of distilled water and then the volume was completed to 100 ml of distilled water autoclaved and stored at 4°C (Prescott *et al*., 2002).

8. Phosphate Buffer Saline (PBS)

It was prepared by dissolving 8 g NaCl, 1.2 g K_2HPO_4 and 0.34 g KH_2PO_4 in amount of distilled water, the pH was adjusted to 7.4 then completes the size to 1000 ml and sterilized by autoclaving then stored in refrigerator (Prescott *et al*., 2002).

2.2.6.1 Specimen collection and bacterial isolation

The urine specimens were collected from patients suffering from urinary tract infections in Kerbala city, using sterile screw capped containers as a mid stream urine. Immediately after collection, these specimens were cultured by streaking on Blood and MacConkey agar, after incubating at 37 ºC for 24 hours, single colony from bacteria that grow on media was recultured three times to obtain pure culture.

Bacterial isolate diagnosis

The bacterial isolates were diagnosed according to cultural, morphological properties and biochemical tests. Morphological and cultured characteristic were performed by observing the shape and colour of the colonies on the selective media. The isolates were also stained by Gram stain to identify Gram positive or Gram negative bacteria.. Biochemical tests were done according to Prescott *et al*. (2002).

1. Catalase test

A drop of 3% hydrogen peroxide was added to a colony or loopful of bacteria transported previously on a slide, a positive result was demonstrated when bubbles of oxygen were released from the bacteria.

2. Oxidase test

A part of colony was transported to a wet piece of filter paper with few drops of freshly prepared 1% redox solution. The development of a violet or purple colour within 10 seconds indicates as a positive test.

3. Coagulase test

Two tubes containing brain heart infusion and a loop full of suspected fresh sample of bacterial isolates were added and incubated at 37 ºC for 24 hours. In a small test tube 0.5 ml of human diluted plasma was added to 0.5 ml of fresh broth culture of the strain to be tested and incubated at 37 ºC and examined for coagulation of plasma after 1, 3 and 4 hours (by tilting the tube gently) for coagulase.

Negative tubes were left over night at room temperature before discarding the growth. Those forming a clot after 24 hours were also counted as positive result.

2.2.6.2 Antibiotic susceptibility:

Bacterial susceptibility to antibiotic was detected according to (Prescott *et al*., 2002).

Muller Hinton Agar:

Medium was prepared according to the manufacturer instruction and were sterilized in the autoclaved at 121°C for 15 minutes.

1. Dilution of bacteria in normal saline then their turbidity was compared with standard McFarland solution 0.5 was approximately $1.5x10^8$ cell/ ml
2. Take amount of 0.1 ml of bacterial solution then spread it onto Muller Hinton agar plate by using L- Glass rod Shape

3. With the sterile forceps the selected discs were placed on the inoculated plate and pressed gently into the agar. Within 15 minutes the inoculated plates were incubated at 37 ºC for 24 hours.

4. After incubation, the diameters of the complete inhibition zone were noted and measured using reflected light and a ruler. The end point, measured to the nearest millimeter, was taken as the area showing no visible growth.

2.2.6.3 Antibacterial activity test:

The method described by (Egorov, 1985) was used with some modifications.

The following steps were used to evaluate the antibacterial activity:

1. Dilution of bacteria in normal saline and their turbidity which were compared with standard McFarland solution 0.5 was approximately $1.5x10^8$ cell/ ml.

2. Take amount of 0.1 ml of solution of each bacterium then spread it onto Muller Hinton agar plate by using L- Glass rod Shape.

3. A sterilized cork borer was used to dig well on the surface on the inoculated plate and then dilute silver nanoparticles by DMSO, 50 µl of silver nanoparticles at concentration (0.01, 0.1, 0.25, 0.5, 1 and 5 mg/ml) and extract of leaf *M. spicata* at concentration (5, 10, 15 and 20 mg/ml) was added to each pore and the plates were incubated at 37°C for 18-24 hr.

4. The inhibitory effect of silver nanoparticles against tested bacteria isolated was determined by measuring the inhibition zones in (mm) around each well.

2.2.7 Antifungal activity of silver nanoparticles and leaf extract of *Mentha spicata*:

Two isolates of fungi were obtained from Department of Biology/ College of Science/ University of Kerbala included *Microsporum canis* and *Trichophyton mentagrophytes*.

Culture media

Sabouraud dextrose Agar

It was prepared by dissolving 65 gm of the medium in 1 liter of distilled water, then autoclaved at 121 ºC for 15 minutes.

The following steps were used to evaluate the antifungal activity:

1. Mixing medium at 45-55 ºC with silver nanoparticles at concentration (0.1, 0.5, 1 and 5 mg/ml), leaf extract of *M. spicata* at concentration (5, 10, 15 and 20 mg/ml) and fluconazole at 50 µg/ ml, then poured in Petri dish.
2. Taking part of fungi and cultured in medium above then incubation at 28 ºC for 7-10 days and compared with control (cultured on a lonely of Sabouraud dextrose agar).
3. Estimation rate of inhibiting diameter of fungi after incubated according to the following equation according to Wang *et al*. (2007).

$$\text{Rate inhibit (\%)} = \frac{\textbf{Diameter of fungi}_{\textbf{control}} - \textbf{diameter of fungi}}{\textbf{Diameter of fungi}_{\textbf{control}}} \times \mathbf{100}$$

2.2.8 Antioxidant activity of silver nanoparticles and leaf extract *M. spicata*:

The free radical scavenging activity using (2,2-azinobis-3 ethylbenzothiazoline 6-sulfonic acid (ABTS)) to the method described by Budrat and Shotipruk (2008) with some modifications.

Solutions:

Solution (1) KOH 0.1M

0.5611 g of KOH was dissolved in small amount of distilled water, and then the volume was completed to 100 ml of distilled water.

Solution (2) Potassium acetate 0.1M

It was prepared by mixing 1.2 ml of glacial acetic acid with amount of distilled water; the pH was adjusted to 4.7 using solution (1). Then complete the size to 100 ml of distilled water.

Solution (3) ABTS 1mM

(2, 2-azinobis-3 ethylbenzothiazoline 6-Sulfonic acid)

It was prepared by dissolving 0.132 g potassium persulfate in amount of potassium acetate 0.1M then adding 0.11 g of ABTS to solution then complete to 100 ml of potassium acetate.

The following steps were used to evaluate the antioxidant activities:

1. A series of dilutions of the concentration of silver nanoparticles was performed between 40-800 μg/ml, leaf extract of *M.spicata* 0.5-3 mg/ml.
2. Dilute solution (3) was prepared by adding potassium acetate solution to give absorption of (0.7± 0.02) at 734 nm then add 3mL of the solution above to 0.3 ml of each concentration of silver nanoparticles.
3. The solution was mixed using a vortex leave at room temperature for 10 minutes.
4. Absorbance was measured at 734 nm estimation rate scavenging ABTS according to the following equation.

$$\mathbf{PI\ (\%) = \Sigma\ 1-(At/Ar) \times 100}$$

At, Ar Absorbance's of sample and ABTS, respectively.

2.2.9 Investigation of the cytotoxic effects of silver nanoparticles

***In vitro* cellular toxicity (blood hemolysis):**

The hemolysis of blood was carried out as described by Bouma (2002) as below:

30 µl of silver nanoparticles solutions at concentration of (50 – 60 µg/ml) were mixed with 0.2 ml of blood (healthy and non smoker). They were mixed gently for 5 seconds then adding 20 ml of normal saline to prevent any hemolysis then centrifuged at 3000 rpm for 10 minutes.

30 µl DMSO with normal saline and blood on the same ratio that used as positive control while the 100% hemolysis was determined by diluting the blood used with 100 fold larger volume of distilled water instead of normal saline. The absorption was measured at 540 nm, Percentage hemolysis was evaluated by equation:

Hemolysis%= (AT – AN) / (A 100% H –AN) x 100%

AT: Absorbance of test solution

AN: Absorbance of normal saline

A100% H: Absorbance of 100 % hemolysis

2.2.10 Statistical analysis

The experiments were conducted and analyzed as factorial experiments with three replications, and compared of differences between the averages by using the less significant difference (LSD).

CHAPTER THREE

3. Results and Discussion

3.1 GC-MS Analysis:

Gas chromatography and mass spectroscopy analysis of compounds were carried out in methanolic leaf extract of *M. spicata* L., Chromatogram of the 18 peaks of the compounds detected was shown in Figure (9).

Shown in table (2) ratio of chemical compound of methanolic leaf of Mentha spicata represents the major compound 2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-, (R) -. It is known that carvone has three double bonds capable of reduction and other compounds can be reducing of silver nitrate (Elmastas *et al*., 2006).

The major compounds of methanolic leaf extract of *Mentha viridis* was reported by Hameed *et al*., (2015) and identified that 3,6-Octadecadiynoic acid, methyl ester.

A total of 19 chemical constituents were identified in the spearmint oil using GC/MS. The main components were carvone (51.7%) and cis-carveol (24.3%), followed by limonene (5.3%), 1,8 cineol (4.0%), cis-dihydrocarvone (2.2%), carvyl acetate (2.1%) and cis-sabinene hydrate (1.0%) (Anwar *et al*., 2010).

Plant extract composition may contribute to reduce silver nitrate and stabilizing of silver nanoparticles (Kumar and Yadav, 2009).

Reported by Kajani *et al*. (2014), *Taxus baccata* are rich in diterpenoids which possibly acts as capping agents and can affect the shape and stability of the nanoparticles.

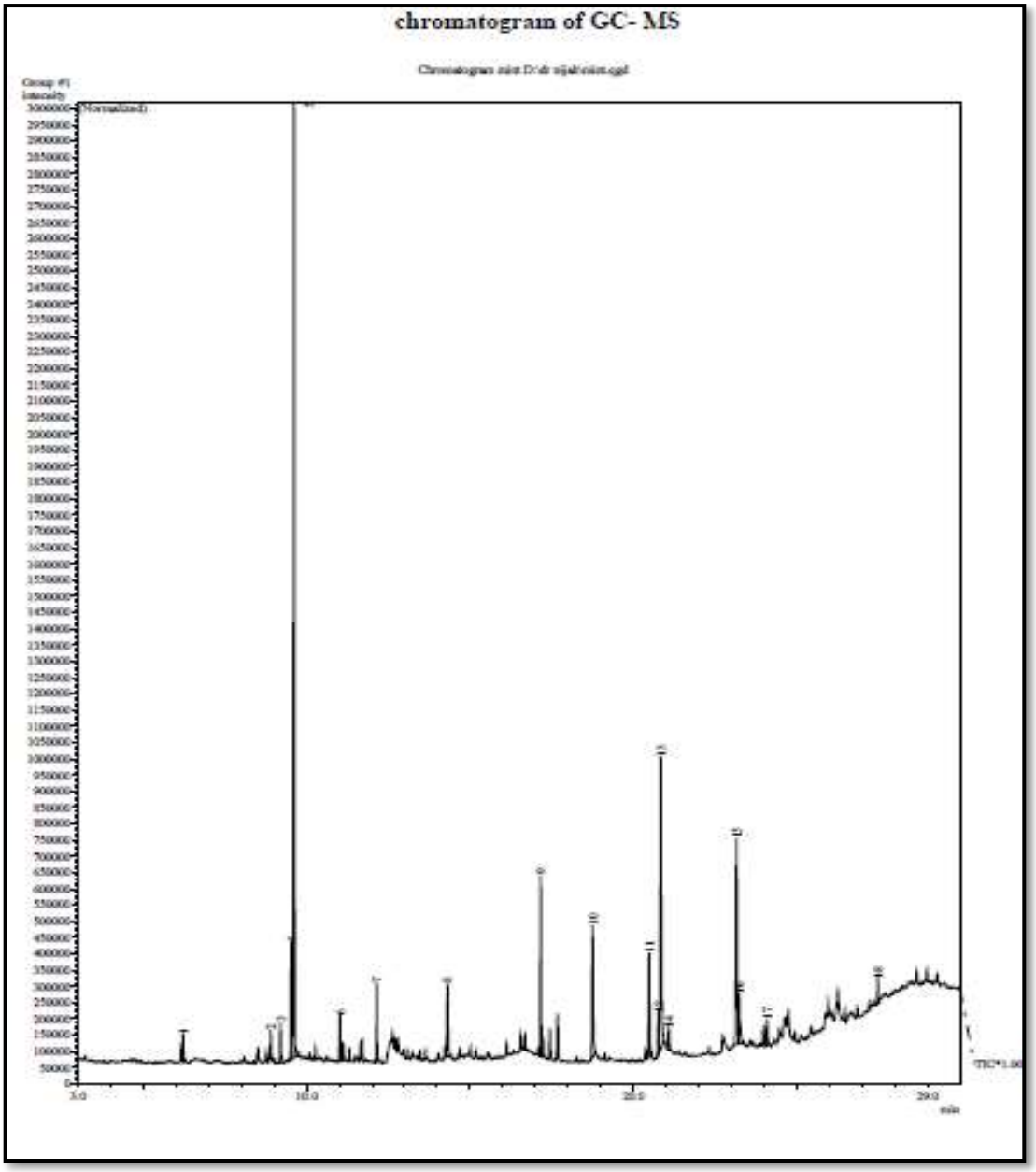


Figure (9): Chromatogram of GC-MS of methanolic leaf extract of *M. spicata*

Table (2): Identified components of methanolic leaf extract of *M. spicata* using GC-MS.

Peak	R.Time	Area	Area%	Name
1	6.221	135328	0.86	Eucalyptol
2	8.901	140897	0.90	Cyclohexanone, 2-methyl-5-(1-methylethenyl)-, trans-
3	9.203	243265	1.55	7-Oxabicyclo[4.1.0]heptan-2-one,3-methyl-6-(1-methylethyl)-
4	9.524	604656	3.86	Pulegone
5	9.622	5313259	33.94	2-Cyclohexen-1-one,2-methyl-5-(1-methylethenyl)-, (R) -
6	11.021	263305	1.68	3,6,10,10-Tetramethyl-1-oxa-spiro[4.5]deca-3,6-dien-2-one
7	12.147	426907	2.73	Caryophyllene
8	14.313	613037	3.92	Phthalic acid, ethyl pentadecyl ester
9	17.162	982378	6.27	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
10	18.756	962633	6.15	l-(+)-Ascorbic acid 2,6-dihexadecanoate
11	20.484	619840	3.96	Phytol
12	20.764	324711	2.07	9,12-Octadecadienoic acid (Z,Z)-
13	20.841	2389533	15.26	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
14	21.082	151650	0.97	Octadecanoic acid
15	23.159	1700226	10.86	2-Methyl-4-(2,6,6-trimethylcyclohex-2-enyl)but-3-en-2-ol
16	23.234	463719	2.96	bis{[(2Z)-3,7-Dimethylocta-2,6-dien-1-yl]oxy}oop(dimethyl)silane
17	24.094	171154	1.09	Bergamotol, Z-.alpha.-trans-
18	27.483	149487	0.95	Tetratetracontane
		15655985	100.00	

3.2 Synthesis of silver nanoparticles:

This experience showed that the silver nanoparticles cannot be synthesized in darkness in case of preparing in cold extract while boiled aqueous extract can be synthesized in darkness after 24 hr.

Aqueous, Ethanol and methanol extract are prepared from leaf of *M. spicata* was used for rapid biosynthesis of silver nanoparticles under bright conditions with direct sunlight radiation.

In addition to chemical synthesis of nanoparticles induced by sunlight, biosynthesis of nanoparticles induced light have been studied by many researches (Dong *et al*., 2004).

Among the various biosynthesis methods, light induced plant extract biosynthesis nanoparticle is opined to be rapid (Zarchi *et al*., 2011) has reported the sun light induced of biosynthesis of silver nanoparticles by *Andrachnea chordifolia* extract, also reported that sun light induced biosynthesis of silver nanoparticles using the aqueous extract of *Cassytha filiformis*. However, the mechanism of biosynthesis of nanoparticles by induced light is not clear (Jena *et al*., 2016).

Recently, silver nanoparticles were synthesized using aqueous garlic extract utilizing solar irradiation. Nanoparticles in this case were synthesized at the end of 15 min when placed in direct sunlight (Rastogi and Arunachalam, 2011).

The oxidation of phenols by pH change to phenolates were depicted as being more readily oxidized than phenols in extract of plant (Zuman and Holthuis, 1988).

The study was quite different from Kajani *et al*. (2014) who used the different change of pH adjusted and temperature after adding silver nitrate to taxus baccata extract, while Kahrilas *et al*. (2013) prepared silver nanopartcles by organ peel extract using microwave- assisted, by *Fraxinus excelsior* leaf extract (Parveen *et al*., 2016).

3.3 Characterization of silver nanoparticles:

3.3.1 UV-Visible spectroscopy:

Brown color change in the reaction due to Plasmon resonance peak was observed for the silver nanoparticles maximum located between 450-550 nm. As shown in figure (10)

The appearance of strong band is the spectral pattern is due to the excitation of the localized surface plasmons which cause strong light scattering by an electric field at a wavelength where resonance occurs, as mentioned Deepa and Ganesan (2013).

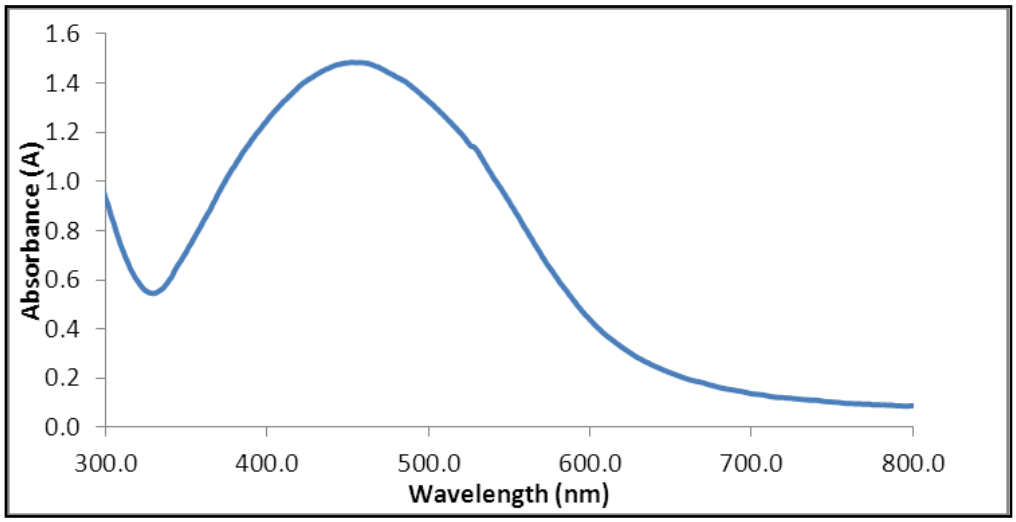


Figure (10): UV-Visible spectra of the synthesized silver nanoparticles

Optimization of biosynthesis of silver nanoparticles using different parameter:

Aqueous silver nitrate solution (1mM, 2mM, 3mM and 4mM) was added to 5%, 10%, 15% and 20% of leaf *M. spicata* extract (with different solvent leaf extract of *M. spicata*), in state light bright (shadow), sun light direct induced synthesis of silver nanoparticles (cold leaf extract of *M. spicata*) while in hot aqueous leaf extract of *M. spicata* can be synthesis in dark. Obtain the best condition of biosynthesis of silver

nanoparticles according to UV-Visble spectroscopy was 1 mM of silver nitrate, 5% of volume extract and no difference significant observed between bright light, sun light direct and dark in aqueous extract, also in methanolic extract between bright light and sun light direct, while in ethanolic extract have difference significant, with respect to time in the sun light direct is fast than bright light and dark.

The U-Visible absorbance spectra of silver nanoparticles recovered from leaf extract of *M. spicata* gave characteristic peak between 450- 550 nm it clearly indicates that the absorption maximum is blue shifted with decrease in the size of the particles. The figures (11, 12, 13, 14, 16, 17, 18 and 19) of the reactions media using different intervals showed the increase in surface plasmon resonance (SPR) band with increasing volume of extract and concentration of silver nitrate.

The shape and size of the silver nanoparticles can be controlled by varying the two parameters of type and amount of extract in the synthesis reaction medium, the large variation in the spectral peaks were attributed to the diversity of extract that reduce the silver nitrate into silver nanoparticles (Kajani *et al*., 2014; Dhanjal and Cameotra, 2010; Oremland *et al*., 2004).

Previous results reported by Chandran *et al*. (2006), increasing amounts of *Aloe vera* extract leads to an increase in population of the spherical particles. Plasmon absorption maximum shifted slightly towards longer wavelengths, which is an indication of particle size increasing, decreasing the volume of extract led to the slower reduction and synthesis of the spherical nanoparticles with more limited size distribution, higher stability in colloids and sharper absorption spectrum; increasing the concentration of silver nitrate above 1 mM caused to rapid reduction of silver nitrate following by agglomeration and precipitation of the nanoparticles (Kajani *et al*., 2014; Mock *et al*., 2002).

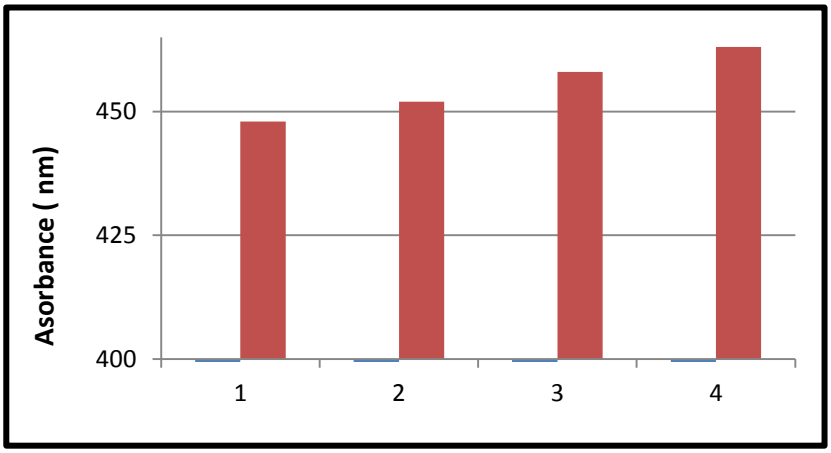


Figure (11): λmax of silver nanoparticles biosynthesized by aqueous leaf extract of *M. spicata*: 5% (1), 10% (2), 15% (3) and 20% (4) with 1 mM of silver nitrate.

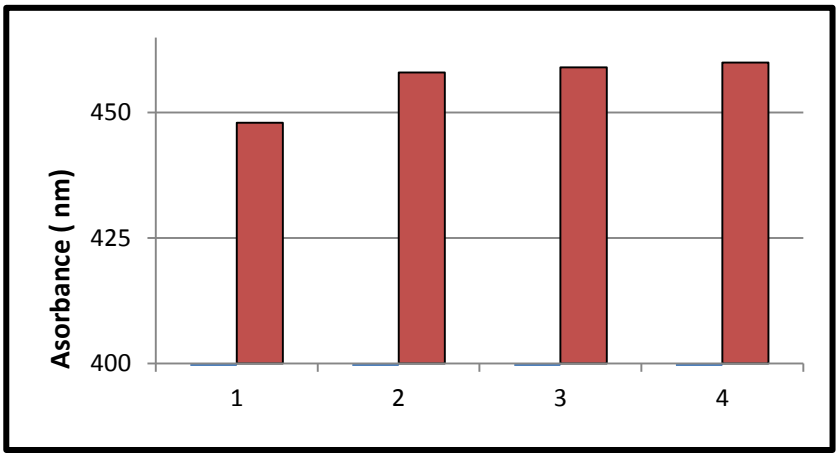


Figure (12): λmax of silver nanoparticles, silver nitrate (1, 2, 3 and 4 mM) with 5% aqueous leaf extract of *M. spicata*.

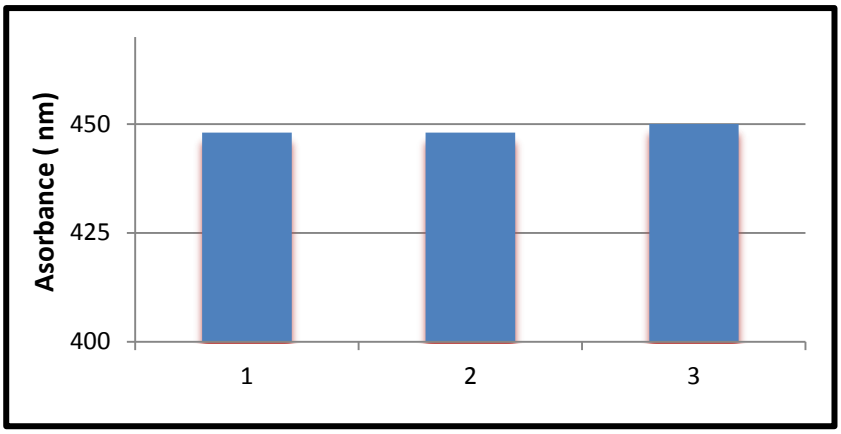


Figure (13): λmax of silver nanoparticles, 1- bright light (shadow), and 2- sun light direct and 3- dark with silver nitrate 1mM with 5% aqueous leaf extract of *M. spicata*.

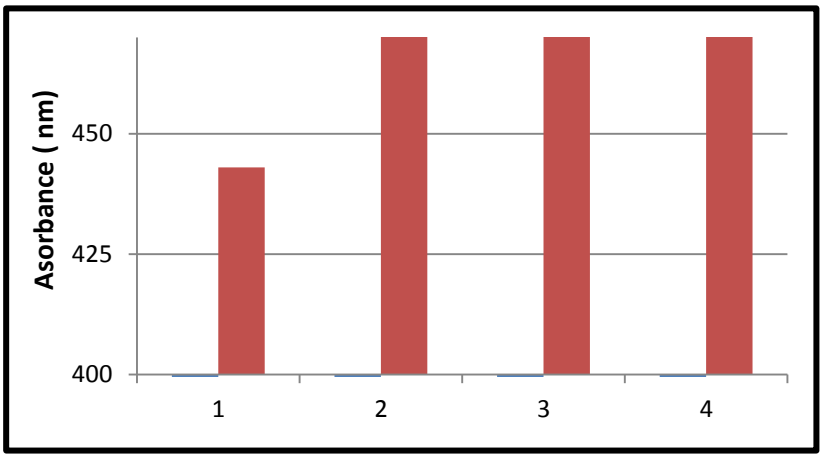


Figure (14): λmax of silver nanoparticles biosynthesized by ethanolic leaf extract of *M. spicata* 5% (1), 10% (2), 15% (3) and 20% (4) with 1 mM of silver nitrate.

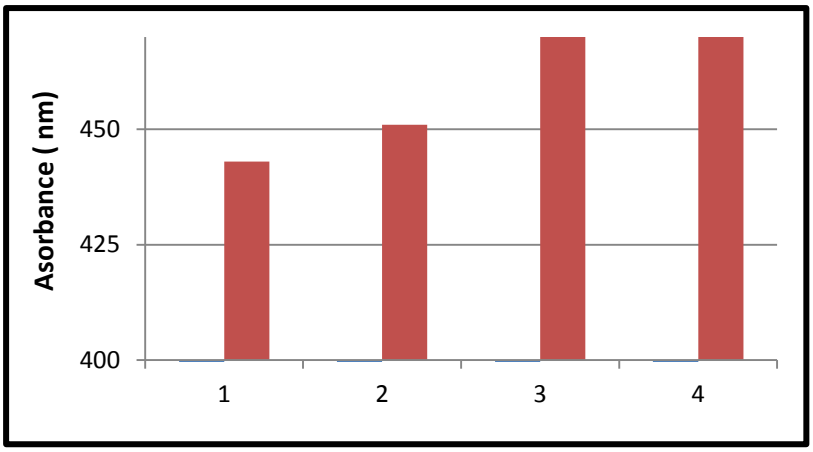


Figure (15): λmax of silver nanoparticles, silver nitrate (1, 2, 3 and 4 mM) with 5% ethanolic leaf extract of *M. spicata*.

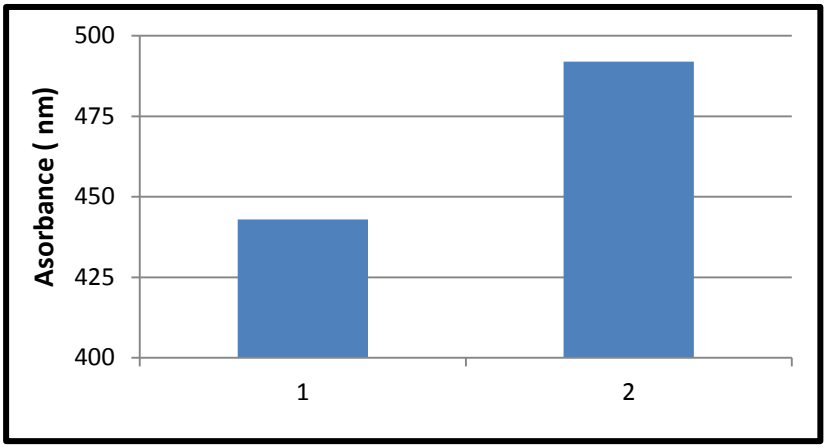


Figure (16): λmax of silver nanoparticles, 1- bright light (shadow) and 2- sun light direct with silver nitrate 1mM with 5% ethanolic leaf extract of *M. spicata*.

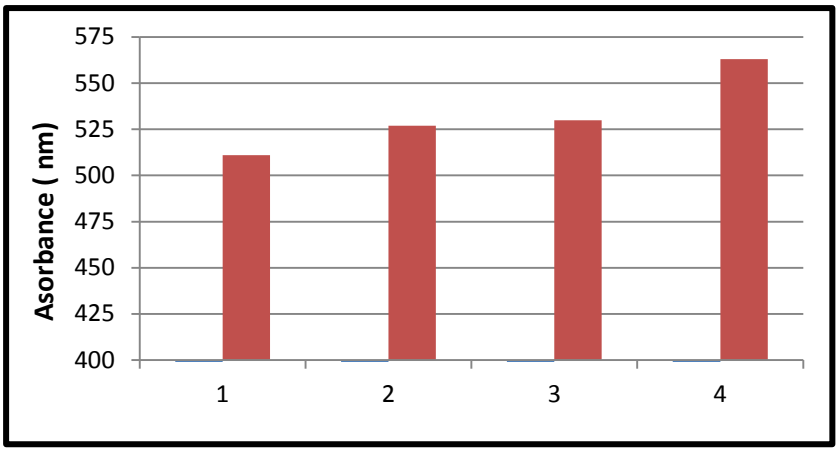


Figure (17): λmax of silver nanoparticles biosynthesized by methanolic leaf extract of *M. spicata* 5% (1), 10% (2), 15% (3) and 20% (4) with 1 mM of silver nitrate.

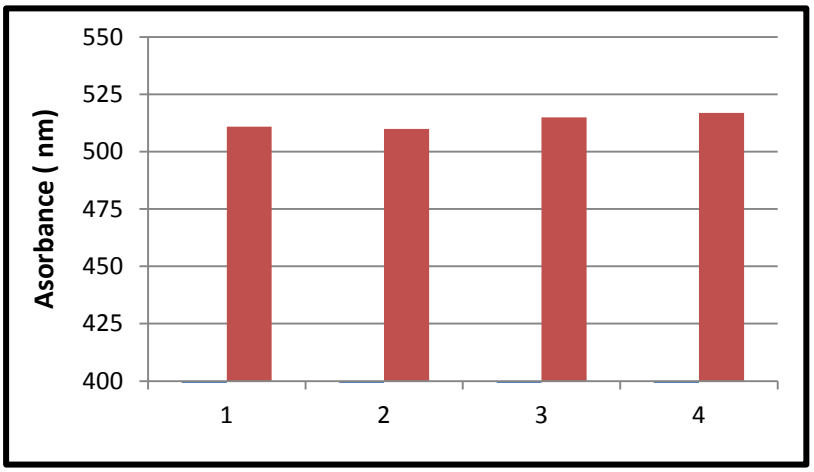


Figure (18): λmax of silver nanoparticles, silver nitrate (1, 2, 3 and 4 mM) with 5% methanolic leaf extract of *M. spicata*.

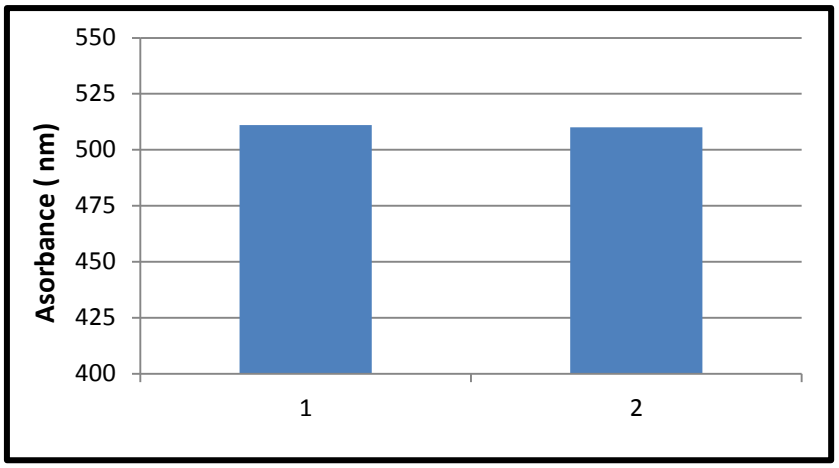


Figure (19): λmax of silver nanoparticles, 1- bright light (shadow) and 2- sun light direct with silver nitrate 1mM with 5% methanolic leaf extract of *M. spicata*.

3.3.2 Fourier transformation infrared spectroscopy (FTIR):

The secondary metabolites are the main factors for the biosynthesis of silver nanoparticles, the plant extract contain phenol, alcohol, amine, carboxylic acid, alkaloids and terpenoids that responsible for reduction and stabilizing silver nanoparticles (Jha *et al*., 2009).

FTIR spectroscopy showed that photochemical analysis of aqueous leaf extract *M. spicata*, it shows prominent bands of absorbance at peaks 3381.33, 2968.55, 1618.33, 1514.17, 1413.87, 1120.68, 1095.60, 887.28 cm^{-1}.

The comparative study of the FTIR spectrum of aqueous leaf extract of *M. spicata* figure (20) and the resulted silver nanoparticles figure (21).

Predicts that a shift in the band from 3381.33 to 3315.74 cm^{-1} which attributed to the stretching vibration of O-H of alcohols and phenols, 2968.55 to 2924.18 cm^{-1} band is attributed to the stretching vibration of C-H aliphatic, 1618.33 to 1610.61 cm^{-1} band is attributed to the stretching vibration of carbonyl (C=O), 1514.17 to 1516.10 cm^{-1} band is attributed to the stretching vibration of C=C, 1413.87 to 1356.00 cm^{-1} band is

attributed to the vibration of C-N, 1120.68 to 1118.75 cm-1 band is attributed to the vibration asymmetric of C-O, 1095.60 to 1091.75cm^{-1} band is attributed to the vibration symmetric of C-O, 887.28 to 827.49 cm^{-1} band is attributed to the vibration of C-C skeletal (Mubarak ali *et al*., 2011).

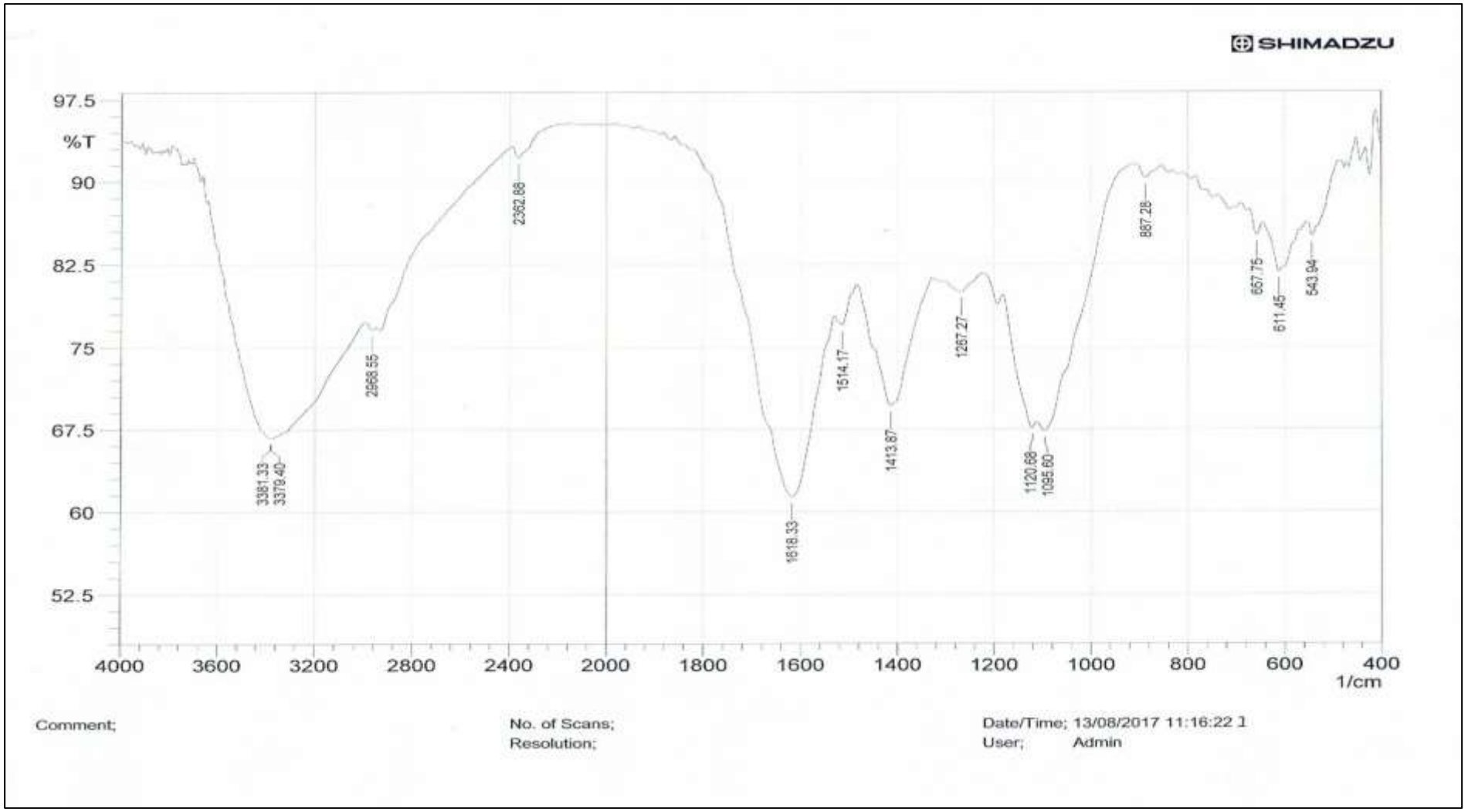


Figure (20): FTIR spectrum of aqueous leaf extract of *M. spicata*.

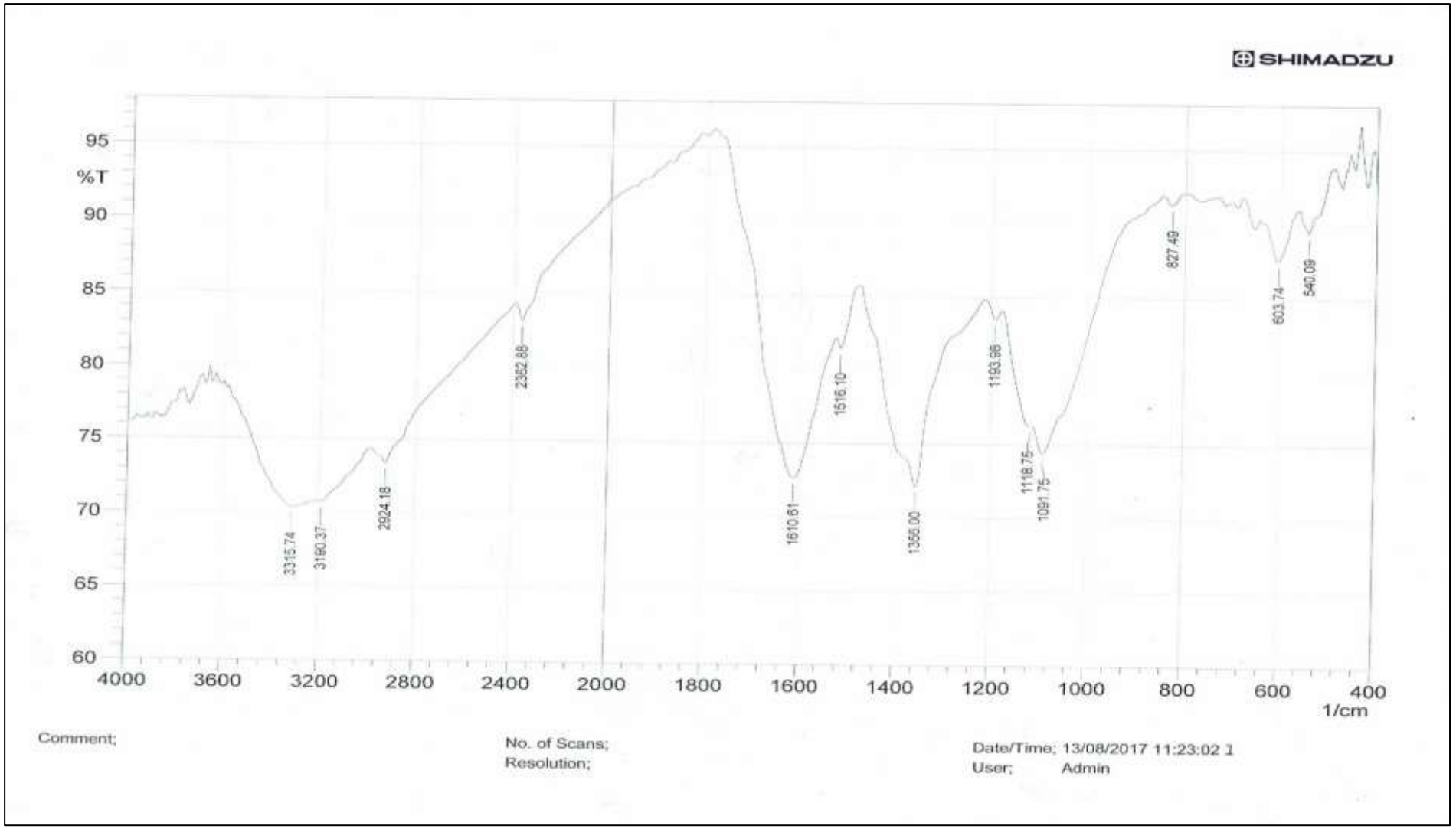


Figure (21): FTIR spectrum of silver nanoparticles synthesized by aqueous leaf extract of *M. spicata*.

FTIR spectroscopy shows that photochemical analysis of ethanolic leaf extract of *M. spicata*, it showed prominent bands of absorbance at peaks 3352.39, 2928.04, 1608.69, 1516.10, 1408.08, 1265.35, 1159.26, 1066.67, 918.15 cm^{-1}.

The comparative study of the FTIR spectrum of ethanolic leaf extract of *M. spicata* figure (22) and the resulted silver nanoparticles figure (23).

Predicts that a shift in the band from 3352.39 to 3745.88 cm^{-1} which attributed to the stretching vibration of O-H of alcohols and phenols, 2928.04 to 2922.25 cm^{-1} band is attributed to the stretching vibration of C-H aliphatic, 1608.69 to 1695.49 cm^{-1} band is attributed to the stretching vibration of carbonyl (C=O), 1516.10 to 1523.82 cm^{-1} band is attributed to the stretching vibration of C=C. The disappearance of the absorbance band at 1408.08 cm^{-1} which attributed to the stretching vibration of C-N, 1265.35 cm^{-1} which attributed to stretching vibration of C-O, 1159.26 cm^{-1} which attributed to stretching vibration of C-O, 1066.67 cm^{-1} which attributed to stretching vibration of C-O, 918.15 cm^{-1} which attributed to stretching vibration of C-C skeletal (Hadjiakhoondi *et al*., 2000).

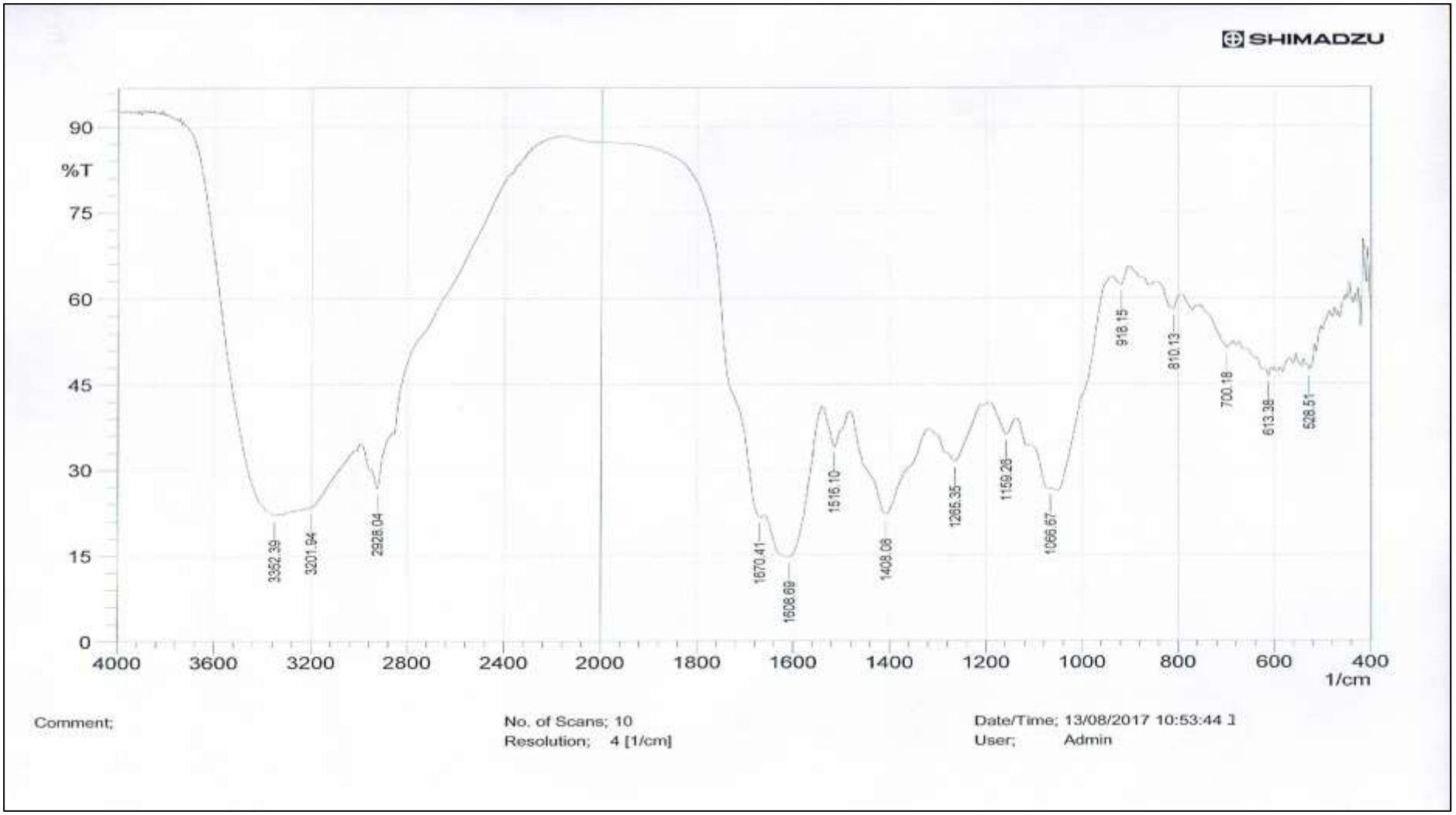


Figure (22): FTIR spectrum of ethanolic leaf extract of *M. spicata*.

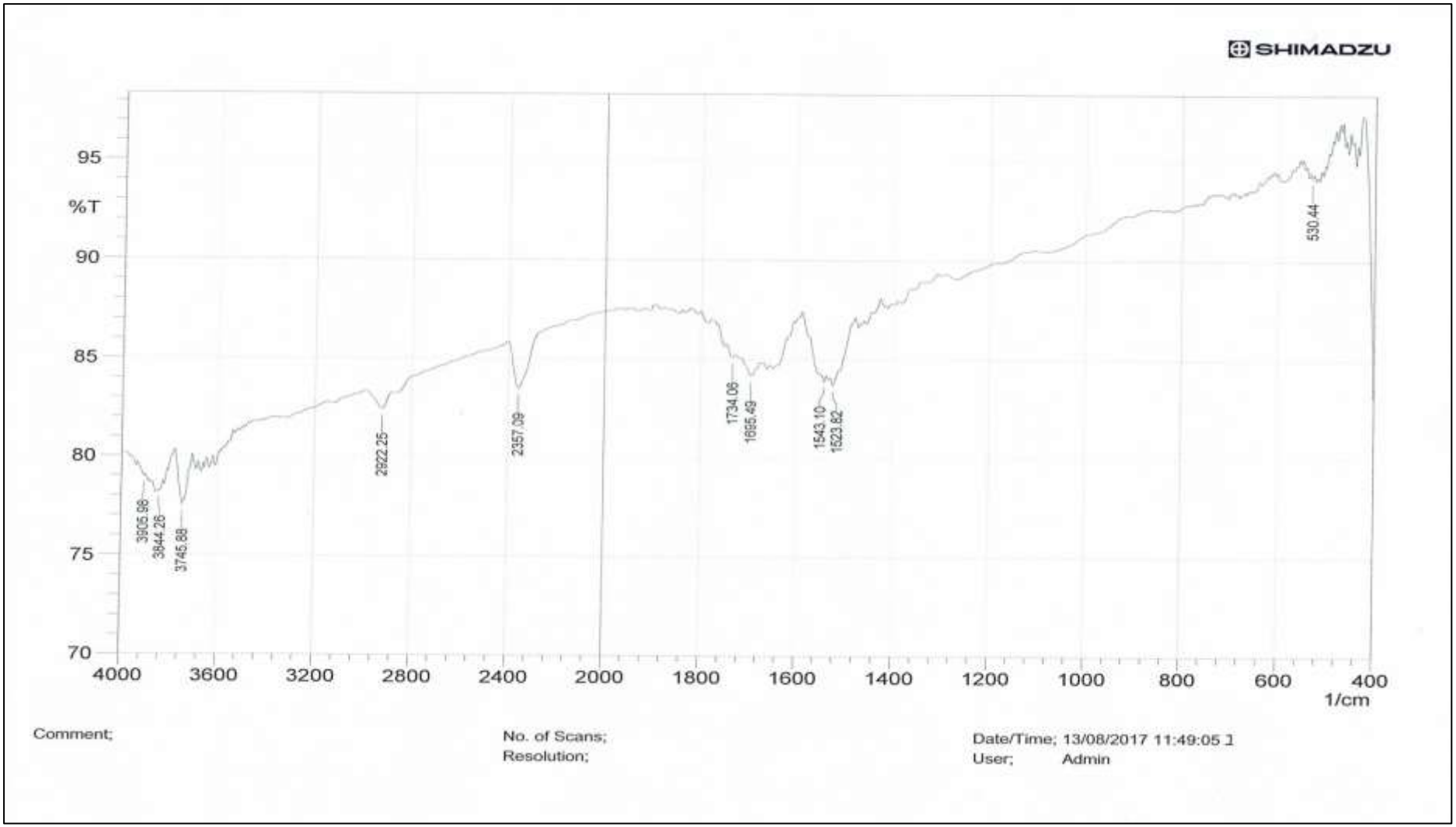


Figure (23): FTIR spectrum of silver nanoparticles synthesized by ethanolic leaf extract of *M. spicata*.

FTIR spectroscopy shows that photochemical analysis of methanolic leaf extract of *M. spicata*, it showed prominent bands of absorbance at peaks 3348.54, 2928.04, 1608.69, 1514.17, 1452.45, 1411.94, 1267.27, 1159.26, 1051.24, 815.92 cm^{-1}.

The comparative study of the FTIR spectrum of methanolic leaf extract of *M. spicata* figure (24) and the resulted silver nanoparticles figure (25).

Predicts that a shift in the band from 3348.54 to cm^{-1} which attributed to the stretching vibration of O-H of alcohols and phenols, 2928.04 to 2924.18 cm^{-1} band is attributed to the stretching vibration of C-H aliphatic, 2858.60 to 2854.74 cm^{-1} band is attributed to the stretching vibration of C-H aliphatic, 1608.69 to 1735.99 cm^{-1} band is attributed to the stretching vibration of C=O, 1514.17 to 1516.10 cm^{-1} band is attributed to the stretching vibration of C=C, 1452.45 to 1458.23 cm^{-1} band is attributed to the bending vibration of C-H, 1411.94 to 1379.15 cm^{-1} band is attributed to the stretching vibration of C-N, 1267.27 to 1163.11 cm^{-1} band is attributed to the stretching vibration of C-O, 1159.26 to 1064.74 cm^{-1} band is attributed to the stretching vibration of C-O, 1051.24 to 1039.67 cm^{-1} band is attributed to the stretching vibration of C-O,815.92 to 875.71 cm^{-1} band is attributed to the stretching vibration of C-C skeletal (Pramila *et al*., 2012).

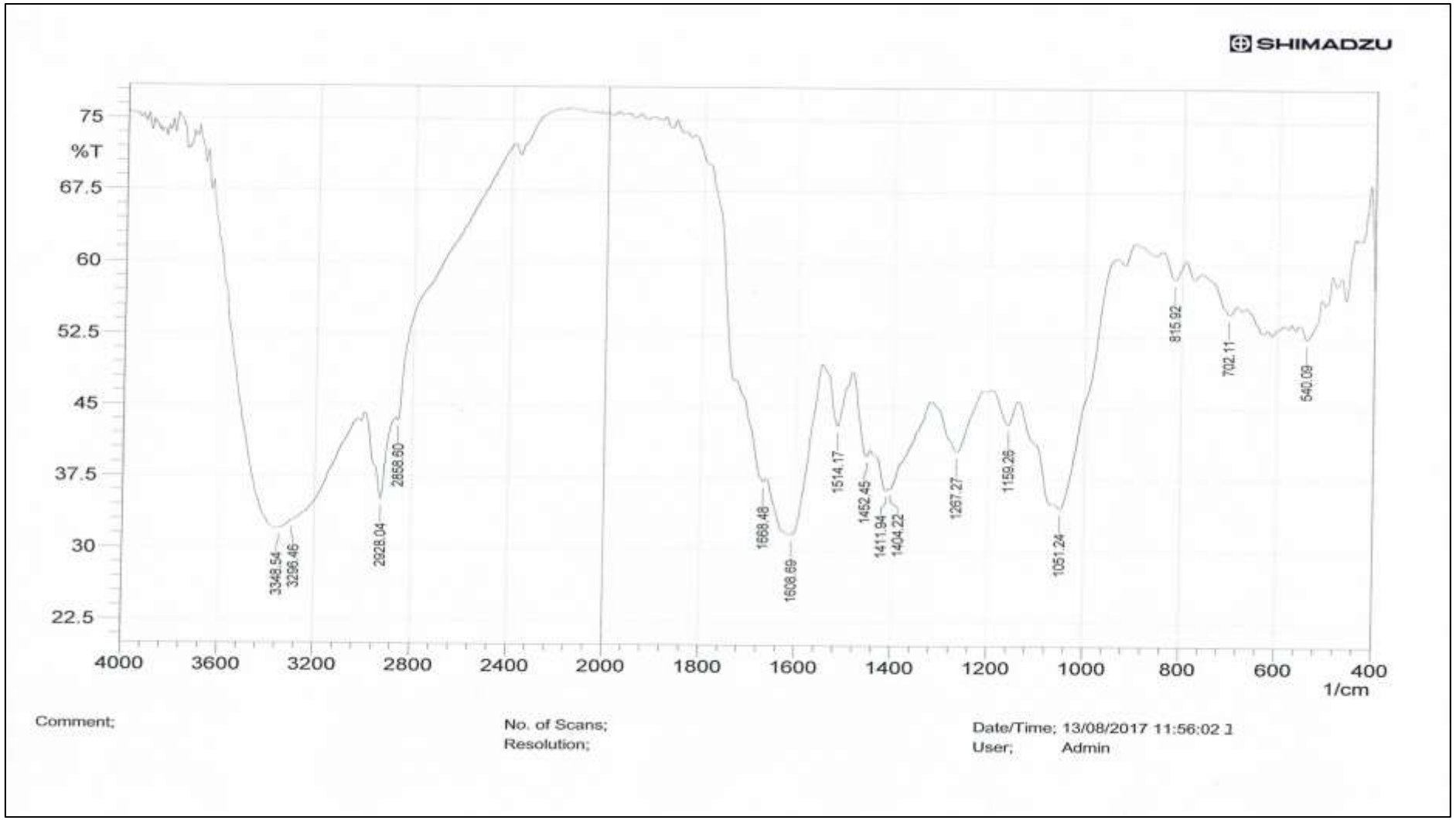


Figure (24): FTIR spectrum of methanolic leaf extract of *M. spicata*.

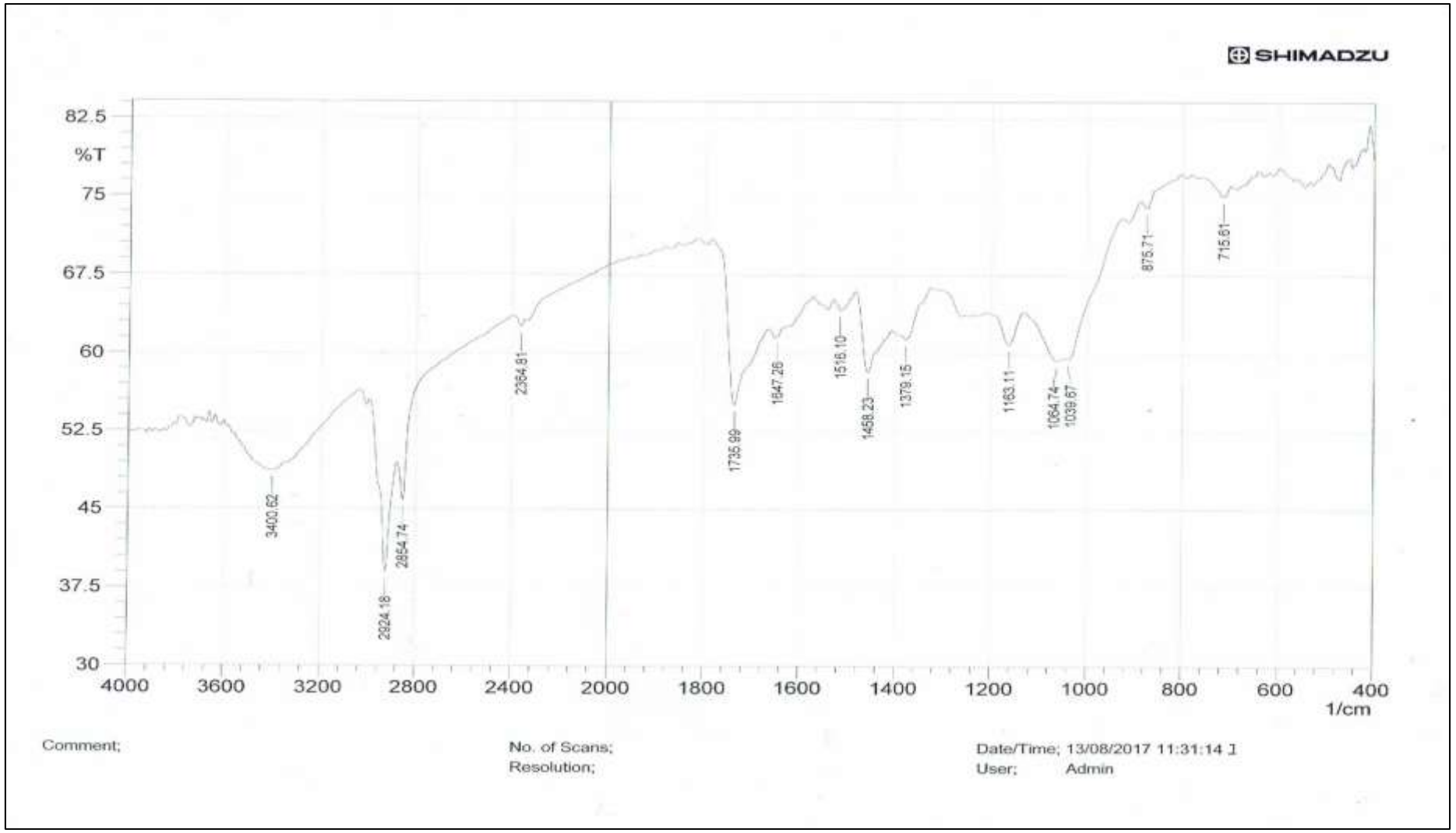


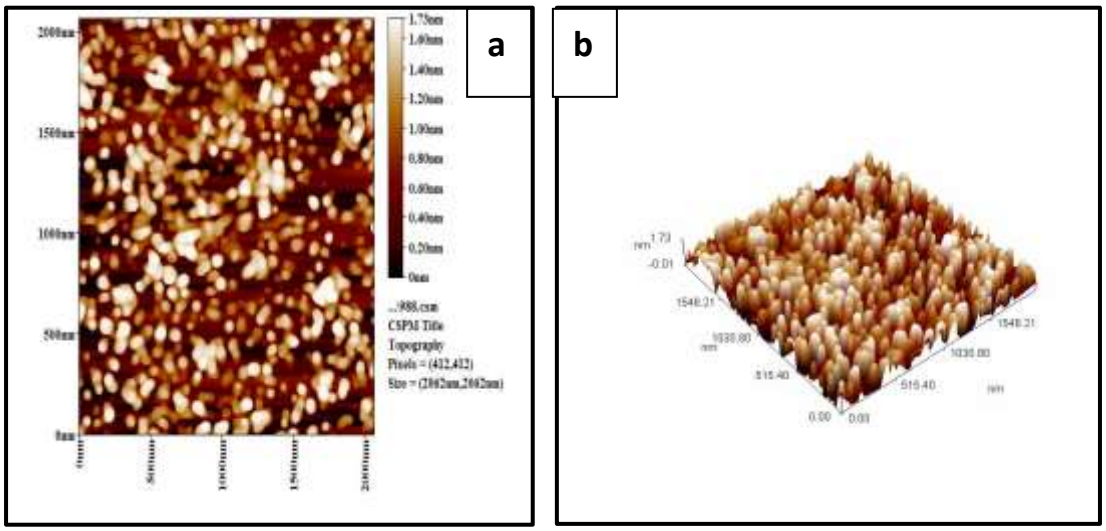
Figure (25): FTIR spectrum of silver nanoparticles synthesized by methanolic leaf extract of *M. spicata*.

3.3.3 Atomic Force Microscopy (AFM):

The topography of the surface was studied using an atomic force microscope. The figure (26-a, 27-a and 28-a) showed two dimensional image of silver nanoparticles showing in clusters of spherical shapes.

The figure (26-b, 27-b and 28-b) showed three- dimensional image of silver nanoparticles revealed a population of homogeneous particles with a regular surface figure.

Show in the table (3,4 and 5) silver nanoparticles the size of particles obtained ranged from 40.00- 70.00 nm, 60.00- 90.00 nm, 65.00- 110.00 nm, in aqueous, ethanolic and methanolic leaf extract of *M. spicata*, respectively, and show the histogram in figure (29, 30, 31).



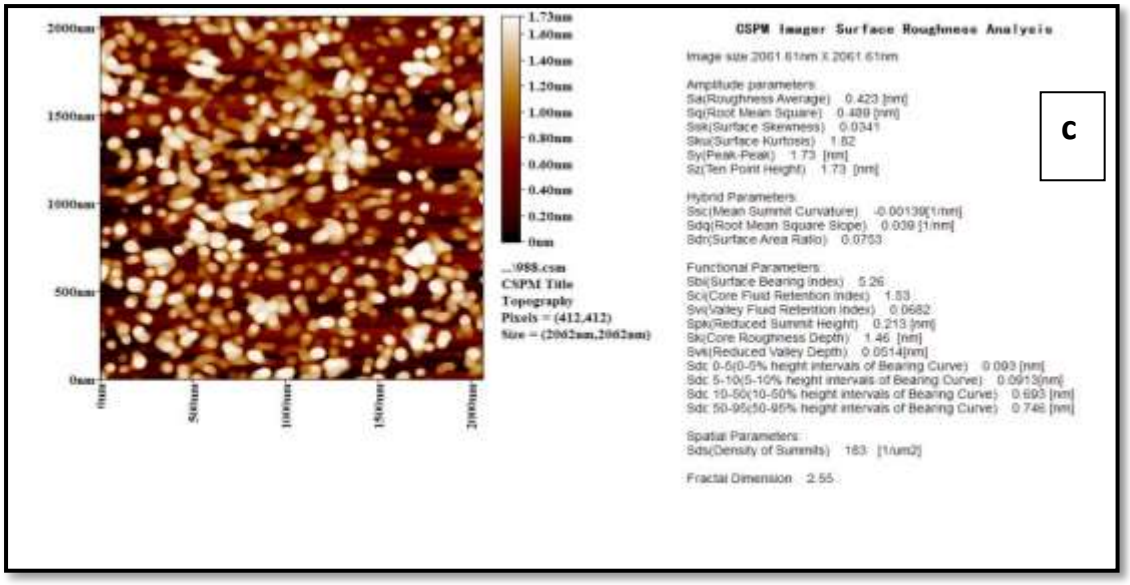
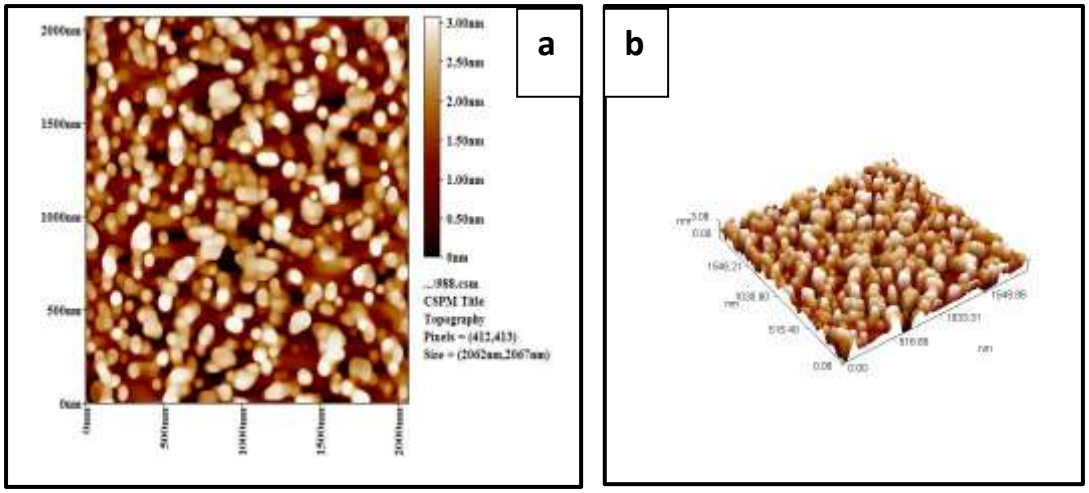


Figure (26): Two-dimensional image (a), three-dimensional image (b), details (c) for silver nanoparticles was synthesized by aqueous leaf extract of *M. spicata*.



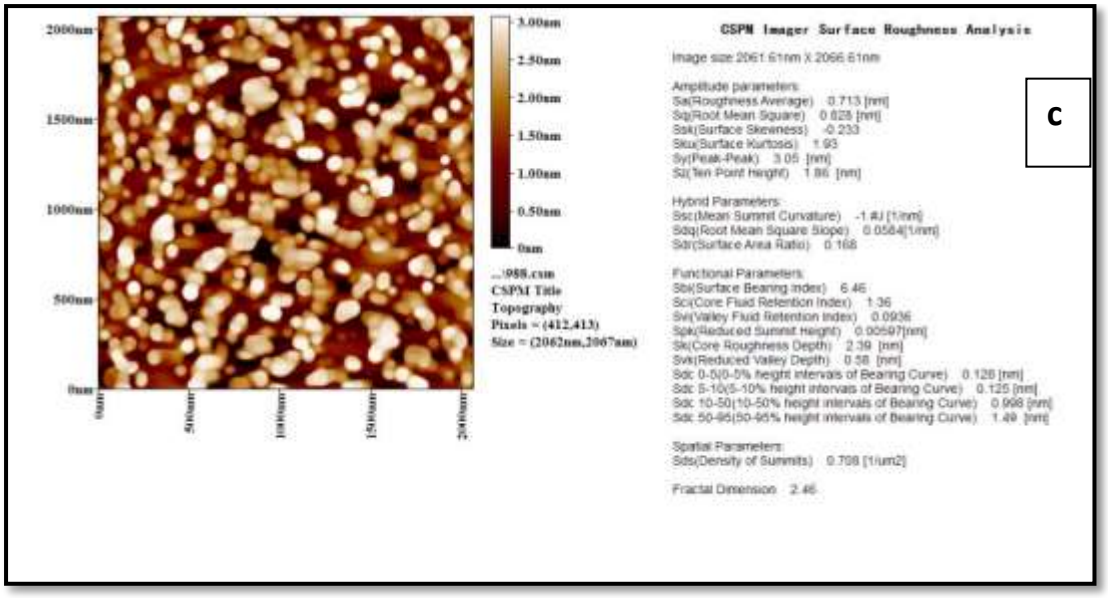
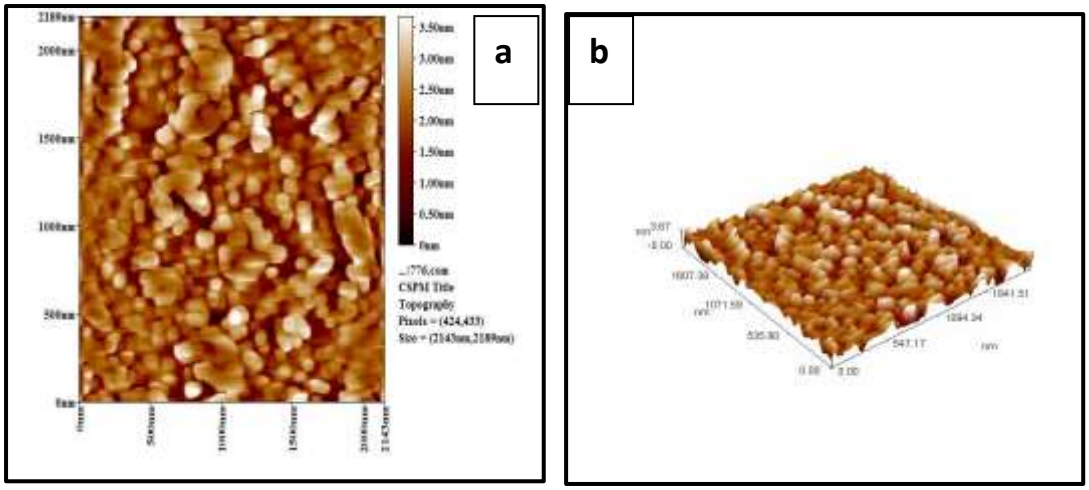


Figure (27): Two-dimensional image (a), three-dimensional image (b), details (c) for silver nanoparticles was synthesized by ethanolic leaf extract of *M. spicata*.



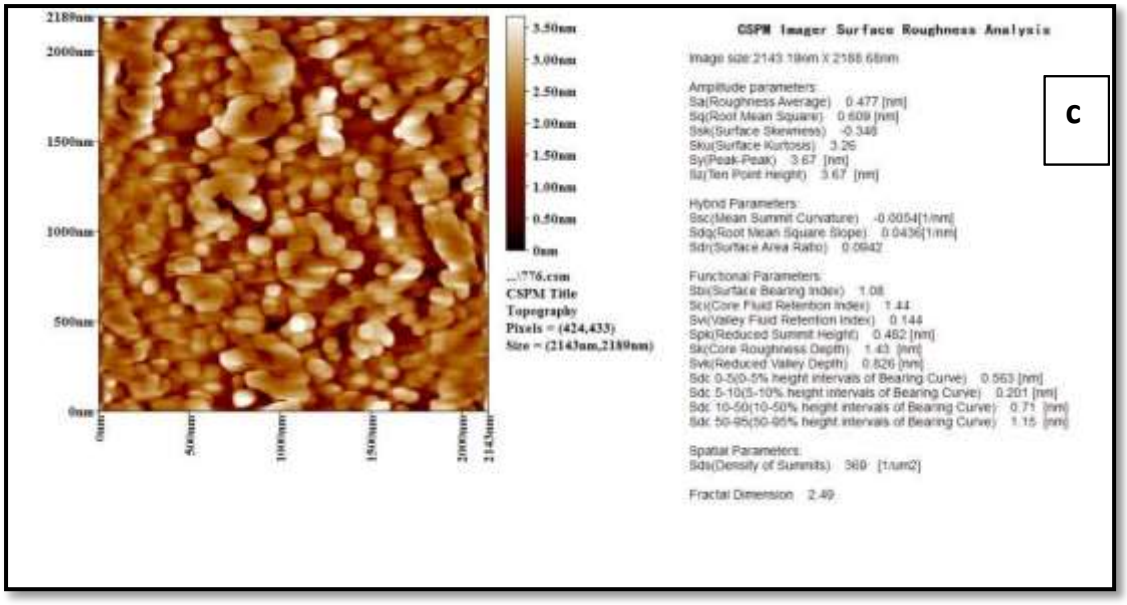


Figure (28): Two-dimensional image (a), three-dimensional image (b), details (c) for silver nanoparticles was synthesized by methanolic leaf extract of *M. spicata*.

Table (3): Diameter and volume of silver nanoparticles synthesized by aqueous leaf extract of *M. spicata*.

Avg. Diameter: 57.51 nm

Diameter (nm)<	Volume(%)	Cumulation (%)	Diameter (nm) <	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)
40.00	2.00	2.00	55.00	16.00	40.40	70.00	14.00	88.00
45.00	13.60	15.60	60.00	14.40	54.80	75.00	12.00	100.00
50.00	8.80	24.40	65.00	19.20	74.00			

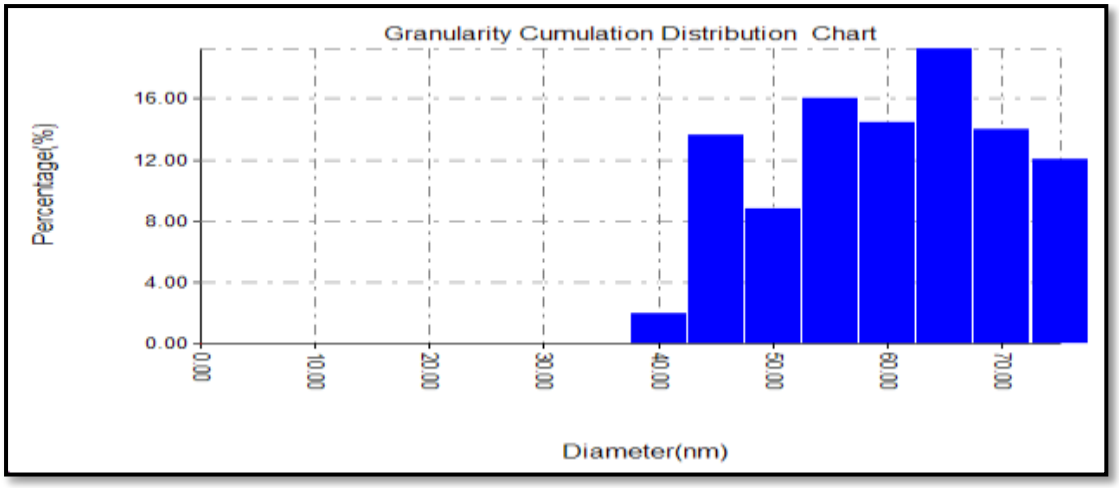


Figure (29): Distribution of pours diameter of the silver nanoparticles.

Table (4): Diameter and volume of silver nanoparticles synthesized by ethanolic leaf extract of *M. spicata*.

Avg. Diameter: 76.40 nm

Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)
55.00	1.49	1.49	75.00	20.15	46.27	95.00	7.46	94.78
60.00	8.21	9.70	80.00	17.16	63.43	100.00	5.22	100.00
65.00	8.96	18.66	85.00	10.45	73.88			
70.00	7.46	26.12	90.00	13.43	87.31			

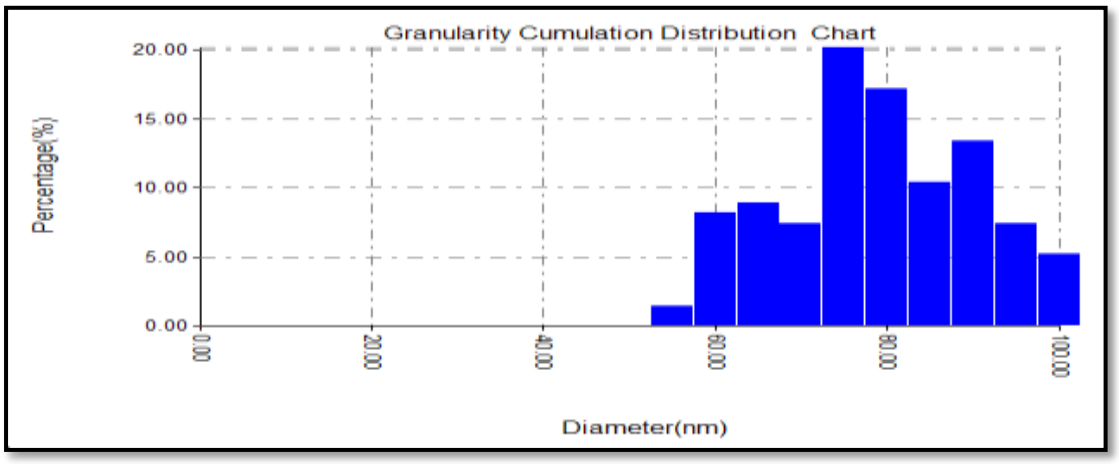


Figure (30): Distribution of pours diameter of the silver nanoparticles.

Table (5): Diameter and volume of silver nanoparticles synthesized by methanolic leaf extract of *M. spicata*.

Avg. Diameter: 91.87 nm

Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)
60.00	2.48	2.48	85.00	6.61	34.71	110.00	13.22	85.95
65.00	6.61	9.09	90.00	11.57	46.28	115.00	4.96	90.91
70.00	6.61	15.70	95.00	7.44	53.72	120.00	4.96	95.87
75.00	4.96	20.66	100.00	5.79	59.50	125.00	2.48	98.35
80.00	7.44	28.10	105.00	13.22	72.73	130.00	1.65	100.00

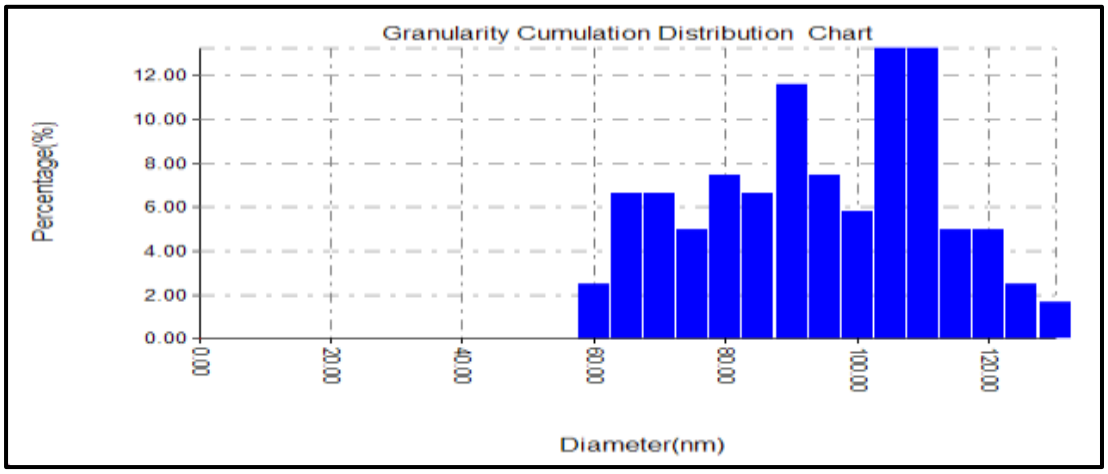


Figure (31): Distribution of pours diameter of the silver nanoparticles.

The table (6) showed the evolutions of the topography of the surface of the silver nanoparticles synthesized by leaf *M. spicata* aqueous, ethanol and methanol extracts. The topography of the surface like roughness average value and root mean square, surface skeweness, surface kurtosis, the value peak-peak in aqueous extract were greater than that synthesized in ethanol and methanol extracts. This indicates a good uniform crystalinity of silver nanoparticles prepared in aqueous solution.

Table (6): Value of surface roughness analysis

		Aqueous ext.	Ethanolic ext.	Methanolic ext.
Amplitude Parameters	Sa (Roughness Average)	0.423 [nm]	0.713 [nm]	0.477 [nm]
	Sq (Root Mean Square)	0.489 [nm]	0.828 [nm]	0.609 [nm]
	Ssk (Surface Skewness)	0.0341	-0.0233	-0.348
	Sku (Surface Kurtosis)	1.8	3.05	3.26
	Sy (peak-peak)	1.73 [nm]	3.05 [nm]	3.67 [nm]
	Sz (Ten point Height)	1.73 [nm]	1.86 [nm]	3.67 [nm]
Hybrid Parameters	Ssc (Mean Summit Curvature)	-0.00139 nm]/ [1	-1#J nm]/ [1	-0.0054 nm]/ [1
	Sdq (Root Mean Square Slope)	0.039 nm]/ [1	0.0584 nm]/ [1	0.0436 nm]/ [1
	Sdr (Surface Area Ratio)	0.0753	0.168	0.0942
Functional Parameters	Sbi (Surface Bearing index)	5.26	6.46	1.08
	Sci (Core Fluid Retention index)	1.53	1.36	1.44
	Svi (Valley Fluid Retention index)	0. 0682	0.0936	0.144
	Spk (Reduced Summit Height)	0.213 [nm]	0.00397 [nm]	0.482 [nm]
	Sk (Core Roughness Depth)	1.46 [nm]	2.39 [nm]	1.43 [nm]
	Svk (Reduced valley Depth)	0.051 [nm]	0.58 [nm]	0.826 [nm]
	Sdc 0-5(0-5% height intervals of Bearing Curve)	0.093 [nm]	0.128 [nm]	0.563 [nm]
	Sdc 5-10 (5-10% height intervals of Bearing Curve)	0.0913 [nm]	0.125 [nm]	0.201 [nm]
	Sdc 10-50(10-50% height intervals of Bearing Curve)	0.693 [nm]	0.998 [nm]	0.71 [nm]
	Sdc 50-95 (50-95% height intervals of Bearing Curve)	0.746 [nm]	1.49 [nm]	1.15 [nm]
Spatial Parameters	Sds (Density of Summits)	183 Um2]/ [1	0.708 Um2]/ [1	369 Um2]/ [1
	Fractal Dimension	2.55	2.46	2.49

3.3.4 Analysis x-rays (XRD):

Diffraction peaks which are indexed by the (111) of the cubic face centered silver. These sharp Bragg peaks might have resulted due to capping agent stabilizing the nanoparticle. Intense Bragg reflections suggest that strong X-ray scattering centres in the crystalline phase and could be due to capping agents. Independent crystallization of the capping agents was ruled out due to the process of centrifugation and redispersion of the pellet in water after nanoparticles formation as a part of purification process. Therefore, XRD results also suggested that the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa. Generally, the broadening of peaks in the XRD patterns of solids is attributed to particle size effects. Broader peaks signify smaller particle size and reflect the effects due to experimental conditions on the nucleation and growth of the crystal nuclei.

XRD analysis showed three distinct diffraction peaks of 27.7°, 35° and 38° at 2θ values indexed to (110) (111), (230) peak and 2θ = 46, 54.2, 56.6, 65.1, 68, and 78 its diffractions at (200) and (023), (150), (113), (220) and (211) planes, which were shown in figures (32, 33 and 34). The crystalline plans of the face centered cubic structure of metallic silver. In the bioreduction process, the average grain size of the silver nanoparticles formed is estimated to be 44.37, 44.98 and 52.900 nm in the extract of aqueous, ethanol and methanol, respectively.

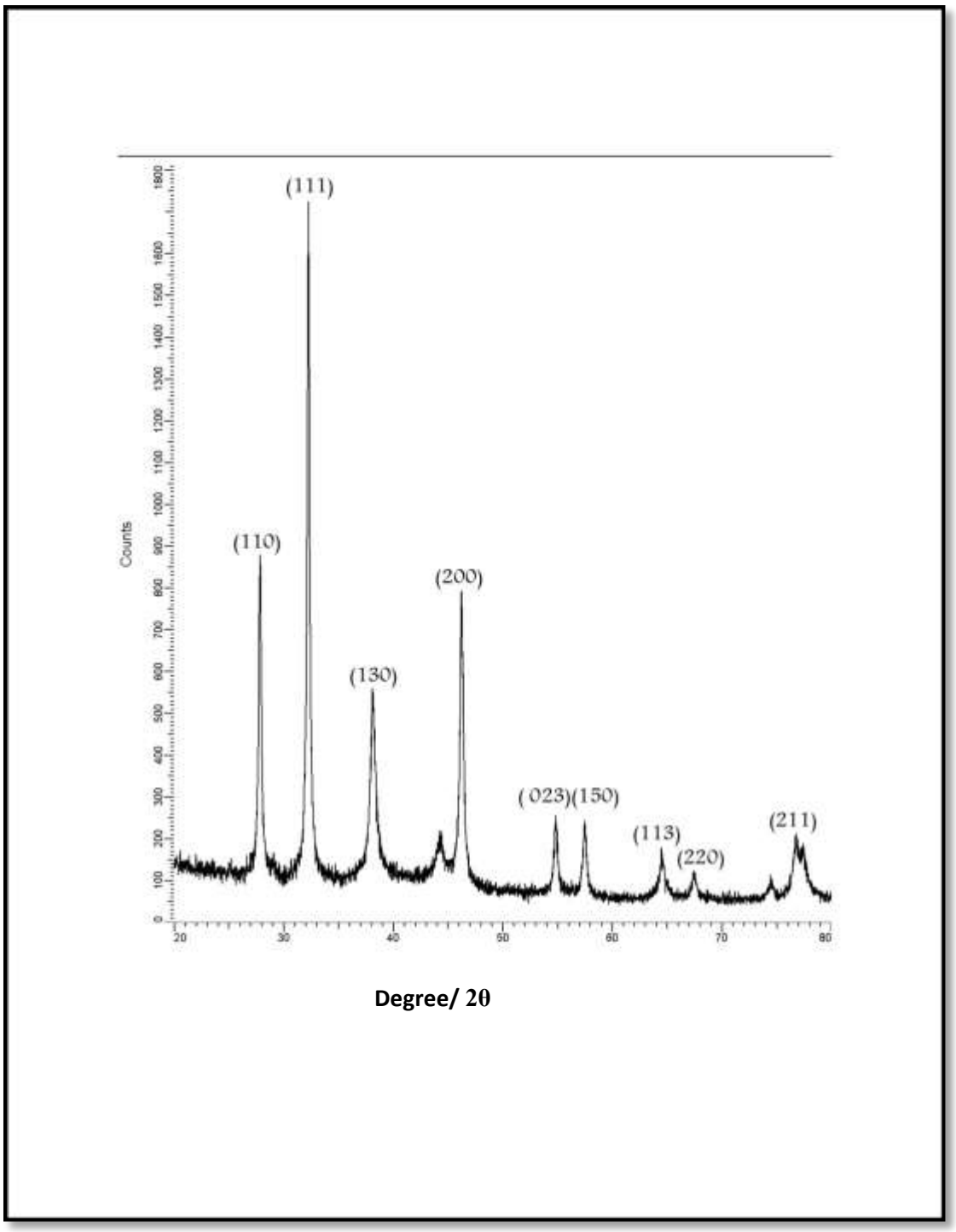


Figure (32): X-ray diffraction showing peak for silver nanoparticles synthesized by aqueous leaf extract of *M. spicata*.

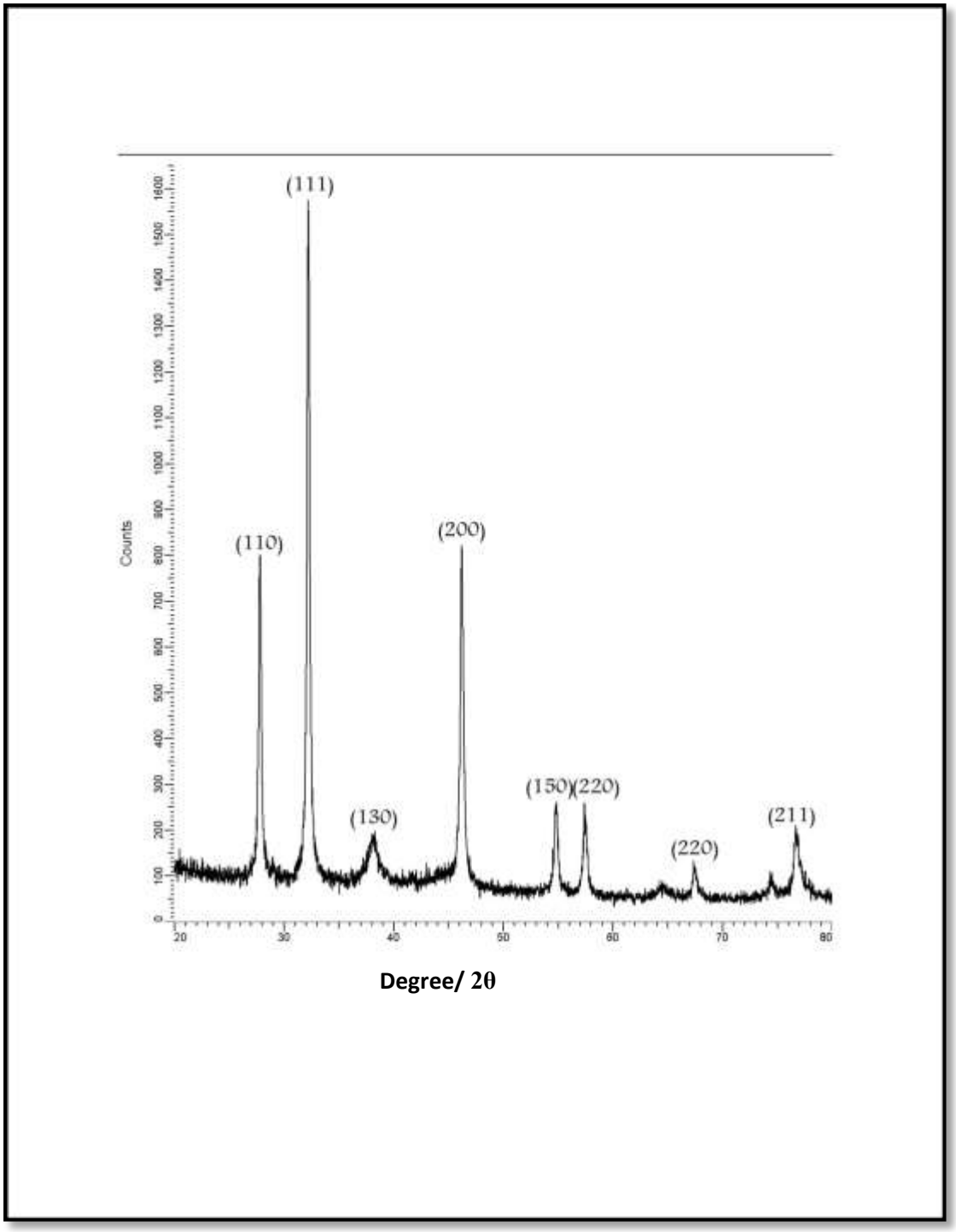


Figure (33): X-ray diffraction showing peak for silver nanoparticles synthesized by ethanolic leaf extract of *M. spicata*.

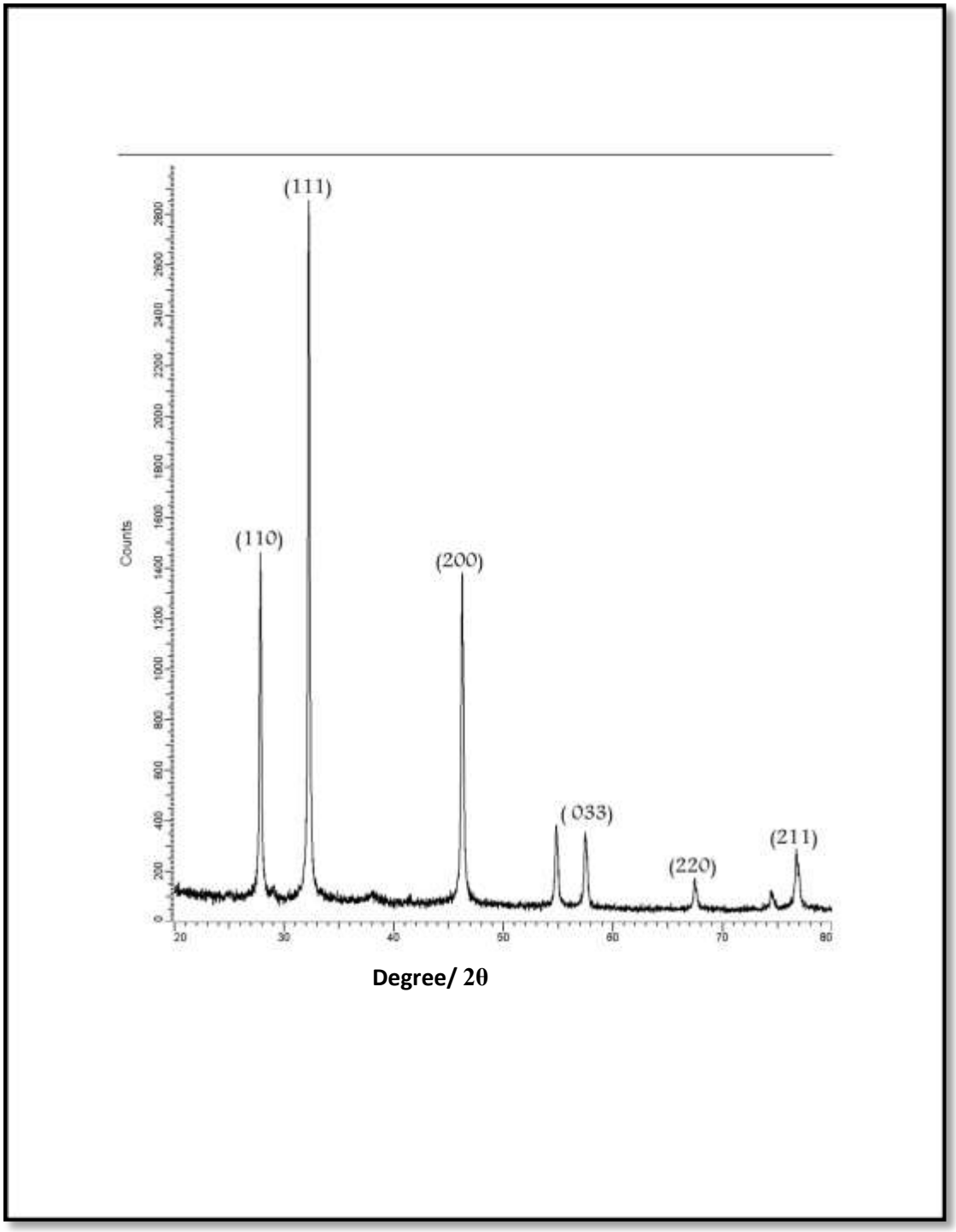


Figure (34): X-ray diffraction showing peak for silver nanoparticles synthesized by methanolic leaf extract of *M. spicata*.

3.4 Biological activity

3.4.1 Antibacterial activity

A- Diagnosis of bacteria

The test 35 urine specimens of patients who suffering from urinary tract infection, bacteria were selected directly from urine specimens by culturing the specimen on general and selective media and after being incubated for 24 h at 37ºC; the growing bacteria were diagnosed biochemically according to methods described by (Abbot, 1999; Winn, 2006; Washington, 2012; Cappuccino and Welsh, 2017) as shown in table (7) and the diagnosis of isolated bacteria were confirmed by APi 20E, Api Staph and Api 20 strep system accomplished according to manufacturer's instructions.

Table (7): Biochemical tests of bacterial isolates

Bacterial isolate	Gram stain	Catalase	Coagulase	Oxidase	Lactose fermentation
S.aureus	+	+	+	-	
E. faecalis	+	-		-	
P.mirabilis	-	+		-	-
E. coli	-	+		-	+

B- Antibiotics susceptibility

The results showed that the isolate *S.aureus* was resist to azthromycin, clindamycin, chloramphenicol, netilmicin, ofloxacin, pencillin and tobramycin, whereas it were sensitive to levofloxacin and Rifampin and it appeared intermediate resistance to erthromycin, *E.faecalis* were resist to azthromycin, erthromycin, clindamycin, ofloxacin, rifampin and pencillin, whereas it were sensitive to Chloramphenicol and Netilmicin, *P.mirabilis* were resist to ceftazidime, tobramycin, chloramphenicol, nalidixic acid, gentamycin, ampicillin, amoxicillin- clavulanic acid , ceftriaxone, netilmicin and aztronam

while sensitive with levofloxacin and amikacin and it appeared intermediate resistance to imipenem and *E. coli* were resist to ceftazidime, gentamycin, ampicillin, amikacin, amoxicillin- clavulanic acid , ceftriaxone, and aztronam while it were sensitive with chloramphenicol, nalidixic acid, imipenem, netilmicin and levofloxacin and it appeared intermediate resistance to tobramycin and *A.baumannii* were resist to ceftazidime, tobramycin, ampicillin, amoxicillin- clavulanic acid, netilmicin and aztronam, while it were sensitive with amikacin, chloramphenicol, gentamycin, levofloxacin and imipenem, and it appeared intermediate resistance to ceftriaxone as shown in table (8).

**A.baumannii* isolate was obtained from public health laboratory/ kerbala.

Table (8): Antibiotics susceptibility of test microorganisms

Antibiotic	***S.aureus***	***E.faecalis***	Antibiotic	***P.mirabilis***	***E.coli***	***A.baumannii***
AZM	R	R	AK	S	S	S
E	I	R	CAZ	R	R	R
CD	R	R	TOB	R	I	R
RA	S	R	C	R	S	S
C	R	S	NA	R	S	--
NET	R	S	GEN	R	R	S
OFX	R	R	AM	R	R	R
P	R	R	AMC	R	R	R
TOB	R	S	LEV	S	S	S
LEV	S	R	IMP	I	S	S
---	---	---	CTR	R	R	I
---	---	---	NET	R	S	R
---	---	---	AT	R	R	R

Bacterial isolates had ability to resist many antibiotics due to they had some mechanisms including: (a) enzymatic drug inactivation; (b) drug target modification; (c) drug permeability reduction; and (d) active efflux of drugs that

allow to survive, or even to actively grow, in the presence of a given antimicrobial agent (Kumar and Varela, 2013).

E. coli, *S.aureus* and *P.mirabilis* were sensitive to levofloxacin and that agreed with Drago, *et al*. (2001) who found that *E. coli*, *S.aureus* and *P.mirabilis* isolate were sensitive to levofloxacin.

E. coli was sensitive to rifampin that disagreement with Thiraviam, *et al*. (2014) who found that *E. coli* was resistant to rifampin.

C- Antibacterial activity of silver nanoparticles and leaf extract of *Mentha spicata*

The antibacterial activites of silver nanoparticles and *M. spicata* leaves extract were evaluted against human pathogenic bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*, *Enterococus faecalis* and *Acinetobacter baumannii* using agar well diffusion method.

The results showed clearly that silver nanoparticles and leaf extract of *M. spicata* which had efficient against these isolates of bacteria as shown in table (9:18).

Table (9): Antibacterial activity of silver nanoparticles against *E.coli*

Concentration mg/ml	**Inhibition zone rate (mm)**			**Average**
	Aq.	Eth.	Meth.	
0.01	14.25	10.25	9.00	11.17
0.1	14.75	11.70	10.00	11.97
0.25	15.75	15.50	11.17	14.14
0.5	17.83	16.83	14.17	16.28
1	18.83	15.83	16.00	16.89
5	21.83	18.67	19.50	20.00
Average	17.21	14.71	13.13	
Control (CTR)	9			

LSD 0.05 (Concentration = 1.222, Extraction = 2.593, Interaction = 3.667)

Table (10): Antibacterial activity of silver nanoparticles against *P.mirabilis*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
0.01	9.33	9.00	9.00	9.11
0.1	10.17	9.67	9.67	9.83
0.25	12.00	11.50	11.50	11.67
0.5	13.00	12.00	12.00	12.33
1	14.00	13.83	13.83	13.89
5	15.83	16.00	16.00	15.94
Average	12.39	12.00	12.00	
Control (GEN)	9			

LSD 0.05 (Concentration = 0.168, Extraction = 2.593, Interaction = 0.504)

Table (11): Antibacterial activity of silver nanoparticles against *S. aureus*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
0.01	9.67	9.17	9.00	9.28
0.1	11.67	10.17	9.00	10.28
0.25	15.67	11.67	10.17	12.50
0.5	16.83	13.00	11.33	13.72
1	18.00	16.33	13.17	15.83
5	18.67	18.00	16.33	17.67
Average	15.08	13.06	11.50	
Control (AZM)	13			

LSD 0.05 (Concentration = 1.136, Extraction = 2.410, Interaction = 3.408)

Table (12): Antibacterial activity of silver nanoparticles against *E.faecalis*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
0.01	9.33	9.33	9.00	9.22
0.1	10.33	10.00	9.33	9.89
0.25	10.50	10.33	9.67	10.17
0.5	12.17	12.17	12.17	12.17
1	18.00	14.67	14.67	15.78
5	22.33	16.83	16.83	18.67
Average	13.78	12.22	11.94	
Control (RA)	8			

LSD 0.05 (Concentration = 0.851, Extraction = 1.806, Interaction = 2.554)

Table (13): Antibacterial activity of silver nanoparticles against *A. baumannii*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
0.01	10.00	9.33	9.00	9.44
0.1	14.17	13.67	10.33	12.72
0.25	15.00	14.67	11.83	13.83
0.5	16.83	15.67	13.67	15.39
1	17.83	17.33	16.17	17.11
5	19.83	19.00	16.83	18.56
Average	15.61	14.94	12.97	
Control (AM)	10			

LSD 0.05 (Concentration = 0.823, Extraction = 1.745, Interaction = 2.468)

The inhibition zone diameter of bacteria isolates increased as the concentration of silver nanoparticles increased to reach to 15.83- 22.33 mm in high concentration of silver nanoparticles 5 mg/ml, but in low concentration 10 mg/ml was 9-14.25 mm. The result of statistical analysis shows that there are differences significant between concentrations. In which 5 mg/ ml exceeds in inhibition zone diameter comparing with other concentration in difference significant.

Different solvents were extract leaf of *M. spicata* and synthesized silver nanoparticles, shows that there are differences significant between of them. In which aqueous extract exceeds in inhibition zone diameter comparing with other solvent in difference significant.

Several privious studies for antibacterial activity of silver nanoparticles, were mentoined by Zhang *et al*.(2013), Silver nanoparticles have antibacterial activity towards the tested pathogenic strains of *E. coli* and *S.aureus*, they also have antibacterial activity towords other pathogenic bacteria (Kokila *et al*., 2016).

studies on the antibacterial activity of three different solvent extracts (water, ethanol and methanol) prepared by previously mentioned from leaf of *M. spicata* which were screened against pathogenic bacteria *S.aureus*,

E.faecalis, *E.coli*, *P.mirabilis* and *A.baumannii* the extracts showed significant antibacterial activities against the test organisms.

The antibacterial activity of any plant depends on the extraction conditions such as type and concentration of the solvent, time and temperature for the extraction process, all these factors effect on the type and the amount of the active material which extracted (Kadhim, 2012).

The inhibition zone diameter of bacteria isolates increased as the concentration of extracts increased to reach to 13-15.67 mm in the high concentration of extract 25 mg/ml, but in low concentration 10 mg/ml was 9 mm.

Antimicrobial activity which may be related to the presence of high content of carvone- rich *M. spicata* oils (Anwar *et al*., 2010).

Table (14): Antibacterial activity of leaf extract of *M. spicata* against *E.coli*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
5	0.00	0.00	0.00	0.00
10	9.00	9.00	9.00	9.00
15	11.83	11.33	10.67	11.28
20	13.83	13.83	13.67	13.78
25	15.17	14.83	15.17	15.06
Average	9.97	9.80	9.70	

LSD 0.05 (concentration = 0.208, extraction = 0.268, interaction = 0.465)

Table (15): Antibacterial activity of leaf extract of *M. spicata* against *P. mirabilis*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
5	0.00	0.00	0.00	0.00
10	9.00	0.00	0.00	6.00
15	9.67	9.33	9.00	9.44
20	11.83	11.00	11.17	11.33
25	13.67	13.67	13.00	13.44
Average	8.83	6.80	8.50	

LSD 0.05 (concentration = 1.745, extraction = 2.253, interaction = 3.902)

Table (16): Antibacterial activity of leaf extract of *M. spicata* against *S. aureus*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
5	0.00	0.00	0.00	0.00
10	9.00	9.00	9.00	9.00
15	12.00	11.67	11.00	11.56
20	14.00	13.33	13.00	13.44
25	15.17	15.67	15.67	15.50
Average	10.03	9.93	9.73	

LSD 0.05 (concentration = 0.259, extraction = 0.334, interaction = 0.579)

Table (17): Antibacterial activity of leaf extract of *M. spicata* against *E. faecalis*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
5	0.00	0.00	0.00	0.00
10	9.00	9.00	9.00	9.00
15	12.00	12.00	12.00	12.00
20	12.50	13.00	12.00	12.50
25	13.83	14.00	13.83	13.89
Average	9.47	9.60	9.37	

LSD 0.05 (concentration = 0.170, extraction = 0.219, interaction = 0.380)

Table (18): Antibacterial activity of leaf extract of *M. spicata* against *A. baumannii*

Concentration mg/ml	Inhibition zone rate (mm)			Average
	Aq.	Eth.	Meth.	
5	0.00	0.00	0.00	0.00
10	9.00	9.00	9.00	9.00
15	10.67	10.33	11.00	10.67
20	13.00	12.17	12.50	12.56
25	14.67	14.00	14.17	12.28
Average	9.47	9.10	9.33	

LSD 0.05 (concentration = 0.213, extraction = 0.275, interaction =0.476)

The results of statistical analysis showed that there were differences significant between concentrations. In which 25 mg/ ml exceeds in inhibition zone diameter compared to other concentration in difference significant. Different solvents were used to extract leaf of *M. spicata*, showed that there were differences significant between of them. In which aqueous extract led to increase in inhibition zone diameter compared to other solvent in difference significant.

The mode of action of these compounds such as polyphenols is generally attributed to poly phenol-protein interactions, though different mechanisms have been suggested including inhibition of microbial enzymes and action on membranes or deprivation of substrates were required for microbial growth (Janecki and Kolodziej, 2010).

3.4.2 Antifungal activity of silver nanoparticles:

The study of antifungal activity against *Microsporum canis* and *Trichophyton mentagrophytes* which causes dermatophytoses, the result in the table (19, 20) showed antifungal activity of silver nanoparticles, whereby the rate of inhibition of diameter of fungi increased along with increase in the concentration of silver nanoparticles, at 5 mg/ml was antifungal 100 percentage of *M.canis* while *T.mentagrophytes* rate of inhibition of diameter at 5 mg/ml was 84.97- 87.27 percentages.

The results agree with Kalaiselvam (2013) reported that having antidermatophytic activity of silver nanoparticles is synthesized by *Aspergillus terreus*, also agreed with this study has been reported the antifungal activity of methanolic leaf extract of *M. spicata* against *T. mentagrophytes* and *M. canis* which are causing dermatophytosis (Mousavi and Kazemi, 2015).

Azole antifungal agents (eg. fluconazole and itraconazole) have been widely used to treat superficial fungal infections caused by dermatophytes (Ghannoum, 2016). These compounds inhibit the sterol 14α- demethylase, a

cytochrome P-450 which involved in ergosterol biosynthesis (Yoshida and Aoyama, 1984).

Table (19): Antifungal activity of silver nanoparticles against *M.canis*

Concentration mg/ml	Percentage of inhibition (%)			Average
	Aq.	Eth.	Meth.	
0.1	51.28	51.60	44.10	49.00
0.5	58.90	55.00	54.00	56.10
1	77.10	76.20	62.40	71.90
5	100	100	100	100
Average	71.80	70.70	62.50	
Control (fluconazole 50 µg/ml)	53.3			

LSD 0.05 (concentration = 4.03, extraction = 4.65, interaction = 0.06)

Table (20): Antifungal activity of silver nanoparticles against *T.mentagrophytes*

Concentration mg/ml	Percentage of inhibition (%)			Average
	Aq.	Eth.	Meth.	
0.1	54.83	50.03	40.00	48.29
0.5	74.83	69.93	64.83	69.87
1	82.27	79.67	79.67	80.53
5	87.27	87.20	84.97	86.48
Average	74.80	71.71	67.37	
Control (fluconazole 5 µg/ml)	48			

LSD 0.05 (concentration = 3.913, extraction = 3.913, interaction = 7.826)

The result is shown in the table (21, 22). It showed antifungal of leaf extract of *Mentha spicata*, the rate of inhibition of diameter of fungi increased along with increase in the concentration of silver nanoparticles; at 20 mg/ml have antifungal 82.3-85.1 percentage of *M.canis* while *T.mentagrophytes* rate of inhibition of diameter at 20 mg/ml was 77.8- 80 percentages.

Table (21): Antifungal activity of leaf extract of *M. spicata* against *M.canis*

Concentration mg/ml	Percentage of inhibition (%)			Average
	Aq.	Eth.	Meth.	
5	38.80	40.03	38.47	39.10
10	60.20	60.03	62.50	60.91
15	70.10	71.97	68.83	70.30
20	82.13	82.47	85.03	83.21
Average	62.81	63.63	63.71	

LSD 0.05 (concentration= 1.160, extraction= 1.340, interaction= 2.320)

Table (22): Antifungal activity of leaf extract of *M. spicata* against *T.mentagrophytes*

Concentration mg/ml	Percentage of inhibition (%)			Average
	Aq.	Eth.	Meth.	
5	42.03	40.97	43.00	40.00
10	57.83	55.27	56.10	56.40
15	66.47	66.83	66.50	66.00
20	77.63	79.70	78.90	78.74
Average	60.99	60.69	61.13	

LSD 0.05 (concentration= 0.829, extraction= 0.958, interaction= 1.659)

The result of statistical analysis revealed that there are significant differences between among studied concentrations.

The *M. spicata* extract and silver nanoparticles appeared variable antifungal activities against test microorganisms (*Microsporum canis* and *Trichophyton mentagrophytes*) in comparison with control (fluconazole), the high concentration of the extract and nanoparticles showed significant differences as antifungal agents in comparison with control.

The mode of action of silver nanoparticles against fungi, some hypotheses arise to validate the antifungal activity of silver nanoparticles. Studies have showed that silver nanoparticles may kill fungal spores by destructing the membrane integrity. In other studies, it has been indicated that silver

nanoparticles may interact with phosphorus and sulphur containing compounds and their interaction may cause damage to DNA and proteins resulting in cell death (Krishnaraj *et al*., 2012).

3.4.3 Antioxidant activity of silver nanoparticles

To evaluate the antioxidant activity of silver nanoparticles for free radical scavenging, efficient of silver nanoparticles to reduction free radical ABTs absorbance to half (IC_{50}) which represented as percentage (PI)%. They were shown in figures (35, 36 and 37) that silver nanoparticles had antioxidant activity 250, 275 and 300 µg/ml comparsion with antioxidant of aqueous, ethanolic and methanolic leaf extract of *M. spicata* that has efficient to reduced the absorbance free radical ABTs to half (IC_{50}) at 1000, 1250 and 1250 µg/ml, respectively.

Due to surface reaction phenomenon and high surface area to volume ratio of silver nanoparticles that absorbed moieties of plant extract (have antioxidant activity) on to the surface and generate a tendency to interact and scavenge free radical (Kumar *et al*., 2011)

Previous studies documented indicate that the silver nanoparticles have antioxidant activity using the ABTs assay: ascorbic equivalent antioxidant capacity that shows at maximam ABTs scavening activity of 64.01% at 100 µg/ml whereas 70.25% at 100 µg/ml of ascorbic (Shanmugam *et al*., 2016).

ABTs assay is an excellent tool for determining the antioxidant activity, free radical absorbed at 734 nm and give bluish green colour, loss of electron of nitrogen atom from ABTs while antioxdant comound or extract has ability to donor hydrogen atom, nitrogen atom in the ABTs capture the hydrogen which lead colourless solution (Leong and Shui, 2002; Marc *et al*., 2004).

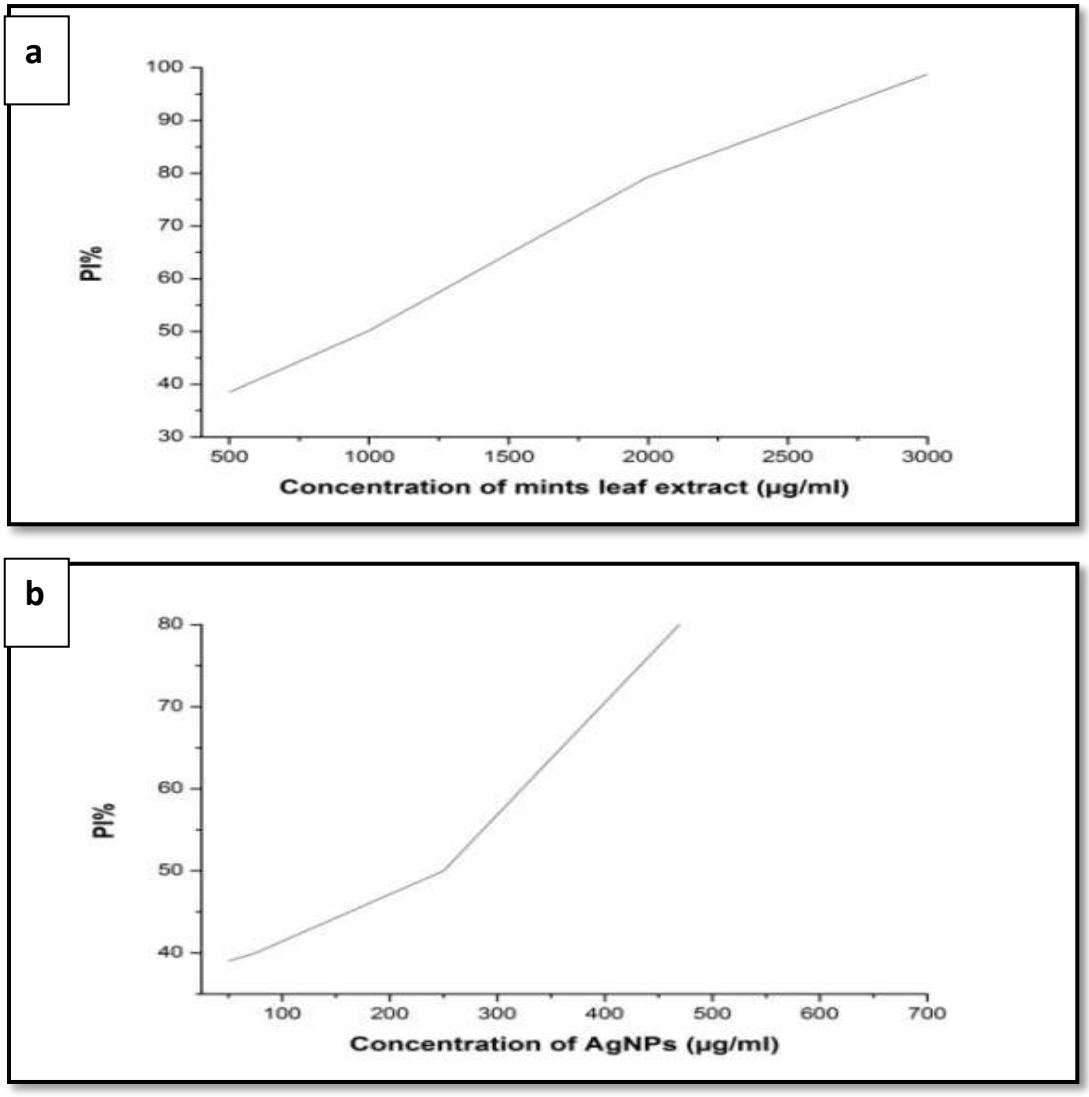


Figure (35): Antioxidant of a = aqueous extract leaf of *M. spicata* and b= Silver nanoparticles

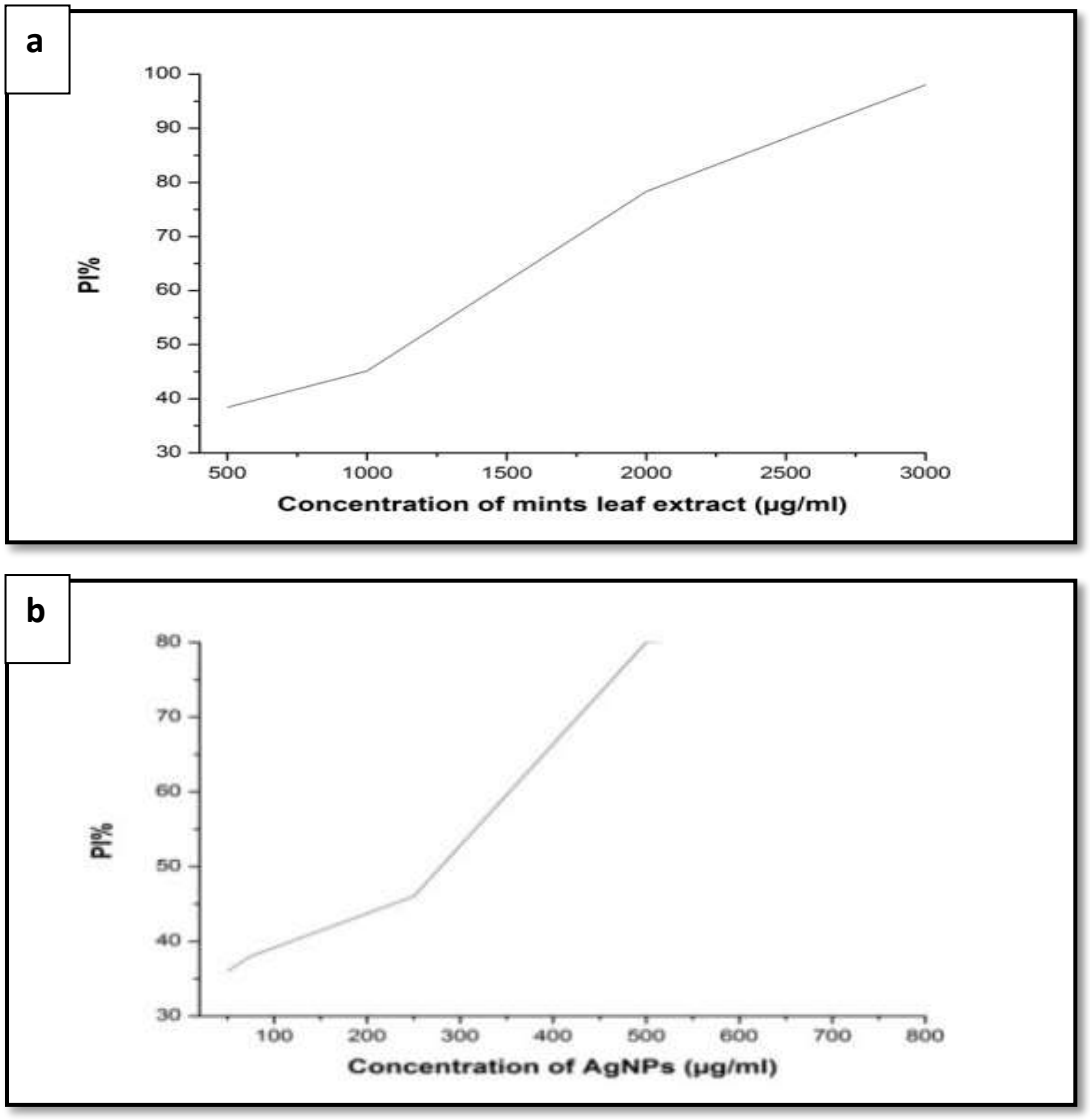


Figure (36): Antioxidant of a= Ethanolic leaf extract of *M. spicata* and b= Silver nanoparticles

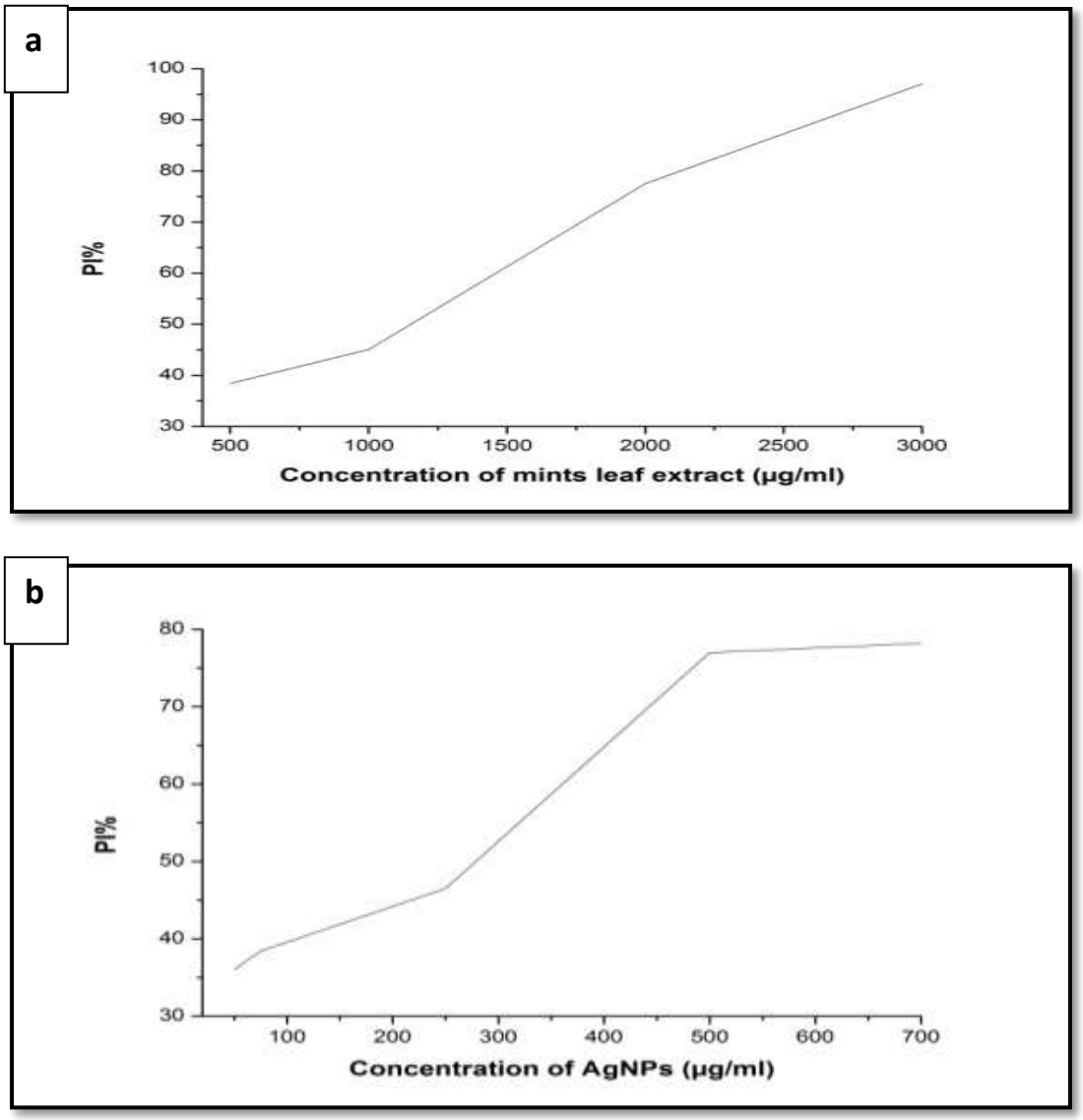


Figure (37): Antioxidant of a= Methanolic leaf extract of *M. spicata* and b= Silver nanoparticles

3.5 Cytotoxic activity of silver nanoparticles

The cytotoxic activity of silver nanoparticles was assayed *in vitro* using human blood erythrocytes (RBCs), table (23) showed the value of absorbance at 540 nm and hemolysis present of silver nanoparticles, in general silver nanoparticles show low RBCs hemolysis percent at 50µg/ml concentration was (2.80%), (3.39%) and (4.84%) with aqueous, ethanolic and methanolic,

respectively; whereas at 60 µg/ml concentraction was (4.50%), (4.24%) and (6.03%) with aqueous, ethanolic and methanolic, respectively.

In our study, we observed an increase in hemolysis (indicating red blood cell destruction) induced by high silver nanoparticles concentrations after direct contact with human blood possibly caused by membrane destruction.

Table (23): Hemolytic activity of silver nanoparticles

Concentration		Absorbance at 540	Hemolysis(%)
AgNPs (Aq.)	50 µg/ml	0.043	2.80
	60 µg/ml	0.062	4.50
AgNPs (Eth.)	50 µg/ml	0.049	3.39
	60 µg/ml	0.059	4.24
AgNPs (Meth.)	50 µg/ml	0.066	4.84
	60 µg/ml	0.080	6.03
Hemolysis 100%	1.186		
Normal saline	0.009		

CONCLUSIONS AND RECOMMENDATIONS

Conclusions and Recommendations

Conclusions

• *Mentha spicata* leaves contain chemical compounds that were reducing agent, and stabilizing for synthesis of silver nanoparticles.

• The optimum of solvent for extraction and biosynthesis of silver nanoparticles was aqueous leaf extract of *Mentha spicata*.

• Cold extract cannot biosynthesize silver nanoparticles unless induced by light.

• Efficient of silver nanoparticles as antibacterial activity against bacteria causing urinary tract infection which are multidruge resistance, also have antifungal activity against fungi causing dermatophytoses that is equal to the effectiveness of the common antibiotic are used (fluconazol), and have antioxidant activity.

Recommendations

• Study biosynthesis for other metal nanoparticles such as zinc, selenium and gold nanoparticles with different plants which it is selection depend on high biological activity (medical plant).

• Biosynthesis of silver nanoparticles by microorganisms.

• Separate of chemical compounds of plant extract and employed on one compound that is related with reduction of silver nitrate.

• Study antimicrobial of silver nanoparticles *in vivo*.

• Study synergism of silver nanoparticles with common antibiotic which are used against bacterial and fungal pathogens.

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APPENDIX

Appendix

Appendix (1): Identification of *S.aureus* isolated from patients by using Api 20 staph system

Test	Result
GLU	+
Fru	+
MNE	+
MAL	+
LAC	+
TRE	+
MAN	+
NIT	+
PAL	+
VP	+
SAC	+
ADH	+
URE	+
XLT	-
MEL	-
RAF	-
XYL	-
MDG	-
NAG	-
LSTR	-

Appendix (2): Identification of *E. faecalis* isolated from patients by using Api 20 srep system

Test	Result
VP	+
HIP	+
ESC	+
PYRA	+
Agal	-
ßGUR	-
ßGAL	-
PAL	**V**
LAP	+
ADH	+
RIB	+
ARA	-
MAN	+
SOR	+
LAC	+
TRE	+
INU	-
RAF	-
AMD	+
GLYG	-

Appendix (3): Identification of *P. mirabilis* isolated from patients by using Api 20 E system

Test	Result
ONPG	-
ADH	-
LDC	-
ODC	+
CIT	-
H2S	+
URE	+
TDA	+
IND	-
VP	+
GEL	+
GLU	+
MAN	-
INO	-
SOR	-
RHA	-
SAC	-
MEL	-
AMY	-
ARA	-

Appendix (4): Identification of *E.coli* isolated from patients by using Api 20 E system

Test	Result
ONPG	+
ADH	-
LDC	+
ODC	+
CIT	-
H2S	-
URE	-
TDA	-
IND	+
VP	-
GEL	-
GLU	+
MAN	+
INO	-
SOR	+
RHA	+
SAC	+
MEL	+
AMY	-
ARA	+

الخلاصة

تصف الدراسة الحالية البناء الحيوي السريع للجسيمات الفضة النانوية باستخدام ثلاثة مذيبات الماء والايثانول والميثانول لأستخلاص اوراق نبات النعناع *Mentha spicata* كعامل مختزل وكعامل مثبت لجسيمات الفضة النانوية.

ادى تعريض محلول نترات الفضة لمستخلص أوراق نبات *Mentha spicata* مما يؤدي إلى اختزال و تغير اللون مما يشير إلى تكون جسيمات الفضة النانوية.

وتمت دراسة الظروف المثلى لبناء الفضة النانوية مثل تركيز نترات الفضة والمستخلص النباتي وتأثير الضوء (كعامل حث لبناء الفضة النانوية). تم الحصول على افضل ظرف لبناء الحيوي للجسيمات الفضة النانوية وكان بتركيز 1mM من نترات الفضة وحجم 5% من المستخلص.

تمت دراسة خصائص جسيمات الفضة النانوية باستخدام مطياف الأشعة المرئية- فوق البنفسجية حيث تظهر القمة عند 450-550 نانومتر و الطيف الأشعة تحت الحمراء يشير إلى وجود جميع المجاميع الفعالة، كما يظهر المجهر القوة الذرية (AFM) متوسط قطر جسيمات الفضة النانوية التي تم الحصول عليها 57.51 و 76.40 و 91.87 نانومتر وتم الكشف بتقنية حيود الأشعة السينية (XRD) الى بناء البلوري لجسيمات الفضة النانوية.

تمت دراسة الفعالية المضادة للبكتيريا لجسيمات الفضة النانوية ضد بكتيريا المقاومة للمضادات الحيوية المسببة لالتهابات المجاري البولية. بتركيز 5 ملغم/ مل قد وصل معدل قطر منطقة التثبيط حوالي 16.33-18.67, 19.5-21.83, 16.83-23.33, 15.83-16, 16.83-19.83 لكل من *Staphylococcus aureus* و *Escherichia coli* و *Enterococcus faecalis* و *Proteus mirabilis* و *Acinetobacter baumannii* على التوالي.

كما تم دراسة الفعالية المضادة للفطريات لجسيمات الفضة النانوية ضد *Microsporum canis* و *Trichophyton mentagrophytes* . بتركيز 5 ملغم/مل قد وصلت نسبة المئوية للتثبيط 100و87.27-84.97 لكل من *Microsporum canis* و*Trichophyton mentagrophytes* على التوالي .

كما تم تقييم جسيمات الفضة النانوية كمضاد للأكسدة وتمتلك نشاط مضاد للأكسدة عند 250 و 275 و300 ميكروغرام / مل بالمقارنة مع مضاد الأكسدة لمستخلص المائي والايثانولي والميثانولي لاوراق نبات *Mentha spicata* التي لها كفاءة في اختزال امتصاص الجذر الحرABTs إلى النصف (IC_{50}) عند 1000 و1250 و 1250 مايكروغرام/ مل على التوالي.

وتم دراسة تأثير الجسيمات النانوية للفضة على تحلل الكريات الدم الحمراء للانسان. عموما جسيمات الفضة النانوية اظهرت تحلل لكريات الدم الحمراء بنسبه قليلة عند تركيز 50 مايكروغرام/ مل تظهر النسبة (2.80%) و (3.39%) و (4.84%) لمستخلص المائي والايثانول والميثانول على التوالي بينما اظهر عند تركيز 60 مايكروغرام/ مل نسبة تحلل لكريات الدم الحمراء (4.50%) و (4.24%) و (6.03%) لمستخلص المائي والايثانول والميثانول على التوالي.

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قســم علــوم الحيـاة

البناء الحيوي لجسيمات الفضة النانوية باستعمال مستخلصات اوراق نبات النعناع *Mentha spicata* ودراسة فعاليتها الحيوية

رســالة مقدمــة إلـــى

مجلــس كليـة العلـوم ــ جامعـة كربلاء

وهـي جـزء مـن متطلبـات نيـل درجـة الماجستيـر علــوم فــي علــوم الحيــاة

من قبل الطالبة

امل جاسم موسى الاسدي

(بكالوريوس علوم/ علوم الحياة – 2015)

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