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Website: Kjar.spu.edu.iq | Email: kjar@spu.edu.iq

# Antibacterial Activity of Copper Nanoparticles Fabricate via Malva Sylvesteris Leaf Extract

**Osama Ismail Haji Zebari**
Akre Directorate Education
Duhok General Directorate
Ministry of Education
Duhok, Iraq
osama.hajin@gmail.com

**Samie Yaseen Sharaf Zeebaree**
Dept. of Medical lab. Technology
Shekhan Technical College of Health
Duhok Polytechnic University
Duhok, Iraq
Samie.ysain@dpu.edu.krd

**Aemn Yaseen Sharaf Zeebaree**
Dept. of Medical lab. Technology
Shekhan Technical College of Health
Duhok Polytechnic University
Duhok, Iraq
aemanzebari2002@gmail.com

**Hardan Ismail Haji Zebari**
Akre Directorate Education
Duhok General Directorate
Ministry of Education
Duhok, Iraq
oihaji84@yahoo.com

**Hadeel Ridha Abbas**
Maternity & Obstetric Department
Sulaimani Technical Institute
Sulaimani Polytechnic University
Sulaimani, Kurdistan Region, Iraq
Hadeel.abbas@spu.edu.iq

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**Abstract**

***A Green reduction of copper ions Cu2+ has been achieved by one-step process and at room temperature utilizing Malva sylvesteris. The extract of Malva Sylvesteris leaf has been identified using qualitative tests to detect the bioactive compounds such as flavonoids, polyphenols, terpenoids and carbohydrates. Characterization of copper nanoparticles was diagnosed by Ultraviolet–visible spectroscopy that confirms the band characteristic for copper nanoparticles in the range of 200–700 nm and the role of Malva Selvesteris leaf extract biomolecules was confirmed by Fourier transform infrared spectroscopy. The crystal shape of nanoparticles was confirmed by X-ray diffraction (XRD) at average (20.96 nm) and the peaks correspond to the face centered cubic structure of cooper metal. Effective antiseptic activity of copper nanoparticles determined by measurement of inhibition zone showed against representative microorganism of bacteria (Gram-positive: Clostridium Staph.aureus;) and (Gram-negative: Escherichia col; Pseudomonas; Klebsiella***

**Keywords:** Fabrication, Nanoparticles, Malva sylvesteris , Microbial susceptibility

## 1. INTRODUCTION

In the last two decades synthesis of nanomaterials, nanoparticles and their applications have improved dramatically due to their unique abilities, physico-chemical properties compared to bulk materials [1]. One of the cheap, available, efficient noble metal that involve in the field of nanotechnology is copper nanoparticles [2]. It has been synthesized by various methods; such as chemical, physical and biological techniques. Parameters and experimental condition that used in such methods to synthesis CuNPs play an significant role of the efficiency of fabricated nanoparticles [3]. In the synthesis copper nanoparticles Cu-NPs, various methods have been used; including sonochemical technique, hydrothermal approach, thermal decomposition, microemulsion method and etc. All mentioned methods had involved toxic chemical compounds and energy demanding routes. Theses lead to synthesized CuNPs less valuable in term of biological and medical applications as well as hazardous to environment [1],[2]. Thus, it needs to an alternative method which is low cost, environmentally friendly, and available row materials [2]. Green syntheses methods could be a good alternative to produce noble metals nanoparticles that their properties correspond to environment friendly, nontoxic and efficient in the chemistry and biology applications [2]. Green reduction chemistry for fabrication of Cu-NPs is an alternative method to replace conventional methods which is using sever (inorganic) reducing agent. A green method has many advantages due to less cost effective, eco-friendly, environmentally and non-toxic solvents are involved in such methods [3]. So in this context recently, many research turn to use plant extracts which is having redox capacity in the synthesis of noble metal nanoparticles, due to it has a vast range of secondary metabolites such as flavonoids, terpenoids, polysaccharides and phenolics. Synthesized of copper nanoparticles by green reduction have widely considerable interest particularly because of the crystal size structure depends on physical and chemical properties and its enormous technological potential [2]

## 2. INTRODUCTION

Copper nanoparticles were synthesized from all the parts of the plant separately like leaf, stem, seed, flower and peels.Malva sylvesteris are as commonly known mallow is a famous plant in medicinal filed. the composition Malva sylvesteris (Mallow) which have a (phenolics and flavonoids) group component of all leaves, flowers, stems and seeds have a suitable and high antioxidant capacity as well as Infusions of it used in traditional medicine for anti-inflammatory and mucilaginous action [4], [5]. And also due to high efficiency of active composition which consist of bioactive compounds consider as good source used in Clinical efficacy of an herbal mouth wash composed in chronic periodontitis patients[5].Among several metal nanoparticles, copper nanoparticles (Cu-NPs) are favorite and attractive due to their specific chemical, physical properties and cheap cost preparation plays as important role in many applications such as in medical field used as a novel as anti-microbial[6]anti-fungal [7]anti-fouling efficiency against growth of algae [8]also as cytotoxicity on human breast cancer cells [3]

The main objective of this study is to synthesis of copper nanoparticles using green and eco-friendly approach without addition any chemicals or undesirable solvent and surfactant and to observe the effect of the Malva Sylvesteris leaf extract (MSLE) as reducing and stabilizing agent in the process of synthesis of Cu-NPs (shape and size). We also confirmed the shapes of formed nanoparticles according to the standard JCPDS (No. 04-0836) data and evaluate antibacterial characteristics of the synthesized copper nanoparticle.

## 3. METHODS AND MATERIALS

### 3.1. Materials

Copper (II) sulfate anhydrase (CuSO4 99.5% purity), was purchased from Scharlau lab Spanish Co. with no further treatment. During the month of April 2019 at the garden of the Shekhan Technical College of Health, a healthy green Malva Sylvesteris Leaves were collected (figure.1A). All Representative microorganisms bacteria (Gram-positive: Clostridium Staph. aureus;) and (Gram-negative: Pseudomonas; E. coli; Klebsiella) are locally isolated in Bacteriology lab of shekhan Technical College of Health and the antibacterial activity evaluated with prepared copper nanoparticles. Bacterial strains were sustained on Nutrient agar slants at 4 0C.

### 3.2. Preparation of Malva Sylvesteris Leaf extracts (MSLE)

Washing Malva Sylvesteris leaves for 10 minutes and dried by microwave for 5 minutes
The (10 g) powders of dry leaves were dissolved in 200 ml deionized water in Erlenmeyer flask 250 ml and boiled at 70 $C^0$ for 2 hrs. the mixture followed by double filtration to remove undesirable compounds in order to obtain yellow solution of MSLE (figure 1.B). The filtrate solution of MSLE was stored in refrigerator -4 0C for further studies.

**3.3. Preparation of 0.1M Copper Sulfate anhydrous Solution** Accurate amount of 0.1M Copper sulphate anhydrous as precursor can be prepared by dissolving 1.595g of CuSO4 in Erlenmeyer flask 200 ml of distilled water were completed and stored in dark place until use (figure1.C).

### 3.4. Green Synthesis of Copper Nanoparticles using MSLE (MS-CuNPs)

Green synthesis of CuNP was achieved in ratio 1:3 which is adding 45 ml of MSLE in Separatory funnel dropwise into 15ml of 0.1M copper sulfate anhydrous aqueous solution in Erlenmeyer flask of 250 ml with stirring for 2 hrs. and at room temperature. When the extract react with the copper ions, the color of the solution show spontaneous change from blue color copper ions to intensive green color. These changes in color of the reaction clearly indicate fabrication of Cu-NPs. The mixture were centrifuged at 4000 rpm for 10 min and the precipitate of nanoparticles then handled with washing by distilled water and left over one day to dry. The next day copper nanoparticles collected (figure 1.D)

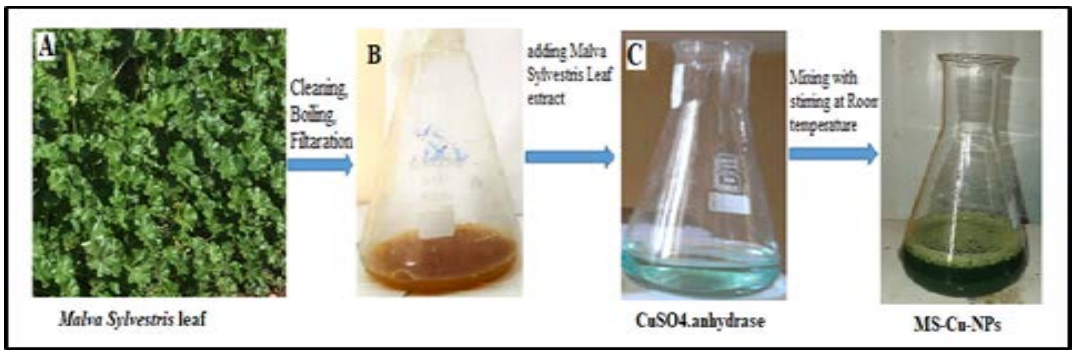


**Figure 1.** Preparation of (A) healthy green Malva Sylvesteris leaves, B) Malva Sylvestris Extract, C) 0.1 M CuSO4 solution (D) MS-Cu-NPs synthesized.

### 3.6 Characterization

#### 3.6.1. Phytochemical Screening – Qualitative Analysis for MSLE

Preliminary phytochemical screening was carried out using standard phytochemical analysis methods for identification of bioactive compounds in Malva Sylvesteris leaf which showed that this plant contains: carbohydrates, reducing sugar, proteins, glycosides, phenols, α-amino acids, tannins, alkaloids, flavonoids, saponins and terpenoids (show Table 1 & Figure 2)

### 3.6.2. Ultra Violet-Visible (UV-Vis) Spectrophotometer

Green reduction of the $Cu^{+2}$ to $Cu^{0}$-NPs achieved by bioreductant aqueous MSLE were monitored by using UV-Vis Spectrophotometer (JENESA 752N Mod.220v). The spectrum analysis of Cu-NPs was scanned from 200 nm to 750 nm and measured after stabilized Color after 24 hours at room temperature using quartz cuvette for the measurement (show Figure 4).

### 3.6.3. Fourier Transform Infrared Spectroscopy (FTIR)

The precipitate of copper nanoparticles and MSLE were characterized by FTIR spectroscopy (Nicolet 6700, ATR) with scanning range 4000–500 $cm^{-1}$ by. The resolution was 4 $cm^{-1}$ with 64 scans and the measurement was done with KBr pellet (show Figure 5a&4b).

### 3.6.4. X-Ray Diffraction (XRD)

Measurement and analysis of XRD pattern of MS-Cu-NPs are shown in (Table 3 & Figure 6) was obtained by using (Philips X'Pert PRO) X-ray diffractometer with 2θ configuration radiation and CuKα (l = 1.540 Å). The diffraction angle was varied in the range of 10–80° while the scanning rate was 0.026/minutes, operating at a voltage of 40 kV and the current of 30 mA.

### 3.6.5. Antimicrobial assay

**Determination of antimicrobial activity for MSLE and MS-Cu-NPs**

This way was used to make the difference between the antimicrobial activity of MSLE and MS-Cu-NPs were investigated by utilizing agar well-diffusion assay by using Mueller-Hinton agar as growth area to measure the sensitivity of tested microorganism against MS-Cu-NPs. The tested microorganisms (bacteria only) on Mueller-Hinton agar plates using sterile cotton were swabbed uniformly then two wells of 6 mm diameter were made using sterile well borer then Twenty microliter of MSLE and (0.1 mM) MS-Cu-NPs solutions was injected into the corresponding well then at 37 $^{0}$C for 24 hours the plates were incubated. The diameter of inhibition zone measured to determine the effect of MS-Cu-NPs as antibacterial (Gram-positive: Clostridium Staph.aureus;) and (Gram-negative: E. col; Pseudomonas; Klebsiella). [4].

### 3.6.6. Antimicrobial activity assessment of MS-Cu-NPs

**Culture medium**

Nutrient stock was done for the elaboration of inoculums of the intended bacteria and Mueller Hinton agar was made for the examination method. The experience bacteria were subcultured using medium of nutrient agar. The tubes include sterilized environment were injected with respective bacterial strains. The bacteria at 37 ± 1 °C for 24 h were incubated then stored in cool condition. The stock cultures were preserved. preparation of bacterial inoculums is made by transmitted a loop of stock culture to the nutrient broth (100 mL) in conical flask (250 mL) then incubated at 37 ± 1 °C (bacteria) and 25 ± 1 °C (fungi) for 24 h before the testing.

**Antimicrobial susceptibility testing**

Antimicrobial sensibility experiment was carried out according to the National Committee for Clinical Laboratory Standards which is by using the agar dilution methods. Measuring of the inhibitory zone diameter is done on the Nutrient Agar for bacteria, with conventional correct well (6mm in diameter) containing particular doses of MS-Cu-NPs. The caliper was used to read the inhibitory zone diameters (MIC), and whole results were approximated to the nearest whole numbers (millimeter) for analysis. [5]

**Minimum inhibitory concentration determination**

Green synthesized MS-Cu-NPs were examined antiseptic activity versus (Gram-negative: *Pseudomonas*; *E. coli*; *Klebsiella* and (Gram-positive: *Staph. aureus; Clostridium*) by agar-well dilution method. investigation of (MIC) of the MS-Cu-NPs versus intended bacteria was

executed by agar well dilution broth method. matching the McFarland (turbidity) standard by standardization of all bacterial dilution, exhibiting a bacterial density of 1.5×108 CFU/mL. therefore, the MHA medium is seeded by 24-hour of microbial strain the media tray. Plates were stand for 10–15 min in order to the culture to absorb in the medium. Four 6-mm-diameter of solidifies agar were produced in each Petri plate and filled with 40 μL (100 μg / mL) of MS-Cu- NPs dissolved in DMSO, antibiotics (Ciprofloxacin) as beneficial control and DMSO as adverse control. MS-Cu-NPs MIC is assessed by preparing with various concentrations starting from 0.5-20 μg / mL Plates were incubated at 37 ° C for 24 hours. Effects were verified based on the inhibition area existence and the area size is measured. The smallest MS-Cu-NP concentration that totally inhibits the development of microorganisms was firm and (MICs) were measured using the recorded approach [6].

## 4. RESULTS

Table 1. Phytochemical Screening – Qualitative Analysis for MSLE

| **Active Comp. in MLE** | **Name of test** | **Methodology** | **Result(s)** |
|---|---|---|---|
| 1) Carbohydrate | Mollisch | 1 ml of Molisch's reagent mix with 1 ml of extract in tube then add $H_2SO_4$ conc. drop by drop | Violet ring Color appeared |
| 2) reducing sugar | Benedict | add 2 ml of Benedict reagent to 1 ml of extract in test tube, and boil for 10 min in water bath | Green precipitate Appeared |
| 3) Protein's | Biuret | 1 ml of 10% NaOH Mix with 1 ml of extract then add 1 ml of 0.7% $CuSO_4$ with shaking | Green precipitate Observed |
| 4) Glycosides | Salkowski | 1 ml of extract mix with Chloroform in 2 ml. shaking then adds 2 ml of $H_2SO_4$ con. carefully | Brown-red ring appeared |
| 5) Amino acid | Ninhydrin | Extract 0.5 ml when boiled with 1 ml of 0.2% Ninhydrin solution | violet color showed |
| 6) Phenols | ---------- | 1 ml of. 2% FeCl3 solution mix 1 ml of extract | black or blue coloration |
| 7) Tannins | Braemer | 1 ml of the extract was poured with drops of 1% lead acetate. | Yellow ppt was formed |
| 8) Alkaloids | Dragendroff | 2 ml of Dragendroff reagent Mix with 1 ml of extract with | Yellowish Turbidity of precipitate forming |
| 9) Flavonoids | Shinoda | 1 ml of extract mix with 1 ml of 40% methanol and shake, then add 1 ml of NaOH | Formation of pink/ Yellowish color |
| 10) Saponin | Frothing | Add 0.5ml of filtrate extract with 5 ml of distilled water and shake well. | establishment foam layer |
| 11) Terpenoids | Liebermann Burchardt | 1 ml of chloroform mixes with 1 ml of extract and evaporated to dryness. Heated about 2 minutes 1 ml of concentrated $H_2SO_4$. | A grayish color indicated |
| 12) steroid | ---------- | A 1 ml extract was blended with 2ml of chloroform and added slightly to the side concentrated $H_2SO_4$. | The bottom layer becomes red and the layer of sulfuric acid is yellow. |

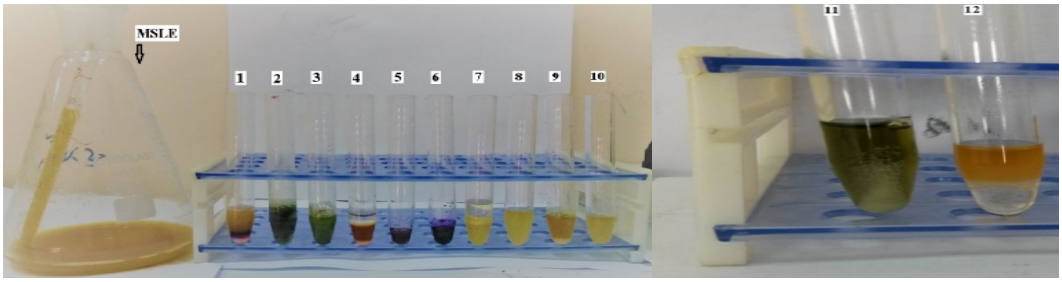


**Figure 1.** Phytochemical Screening – Qualitative Analysis for MSLE

## 5. DISCUSSION

### 5.1. Qualitative Phytochemical Screening of Malva Sylvesteris Leaf extract

In Table 2 The results of qualitative phytochemical analysis of the Malva Sylvesteris (MSLE) revealed different color belong to the bioactive compounds that found in leaf of the Malva Sylvesteris plant which confirmed by known chemical tests in table.1 which proved the presence of the bioactive compounds such as carbohydrates, flavonoids, alkaloids, glycosides, tannins, saponins, phenols and terpenoids. Active groups in these compounds such as carbonyl, hydroxyl and amine will play an important role as capping and stabilizing agent to reduce Cu2+ to Cu0.

Table 2. Qualitative Phytochemical Screening of Malva Sylvesteris Leaf extract

| Active Comp. in MLE | Name of test | Result(s) | Color |
|---|---|---|---|
| 1) Carbohydrate | Mollisch's Test | (+ve) | Violet ring Color appeared |
| 2) reducing sugar | Benedict test | (+ve) | Green precipitate appeared |
| 3) Protein's | Biuret test | (+ve) | Green precipitate Observed |
| 4) Glycosides | Salkowski Test | (+ve) | Brown-red ring Appeared |
| 5) Amino acid | Ninhydrin test | (+ve) | violet color appeared |
| 6) Phenols | ---------- | (+ve) | blue or black coloration |
| 7) Tannins | Braemer's test | (+ve) | A yellow precipitate was formed |
| 8) Alkaloids | Dragendroff Test | (+ve) | Yellowish Turbidity of precipitate Forming |
| 9) Flavonoids | Shinoda test | (+ve) | Formation of pink/ Yellowish color |
| 10) Saponin | Frothing test | (+ve) | formation of foam layer |
| 11) Terpenoids | Liebermann Burchardt test | (+ve) | A grayish color indicated |
| 12) steroid | ---------- | (+ve) | The bottom layer becomes red and the layer of sulfuric acid is yellow |

(+ve) present active compound in MSLE.

### 5.2. Ultra Violet-Visible (UV-Vis) Spectrophotometer

The addition of MSLE extract to Copper (II) sulfate anhydrase solution resulted in color changing from blue to intense green due to formation of Cu-NPs and the maximum absorption peaks of copper nanoparticle at 560 nm. This is happened due to surface Plasmon absorption of copper nanoparticles which is collective oscillation of free electrons from conduction band move to exciton band by the incident electromagnetic radiation. And there were two band appear in red shift 250 nm (band I) and 310 nm (band II) assigned to cinnamoyl system of benzoyl type phenolic compounds (show figure .3)

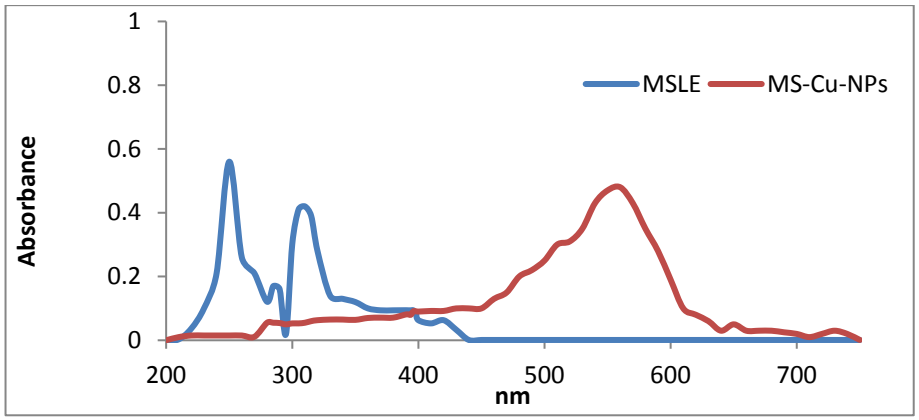


**Figure 3.** UV-VIS Spectra of MSLE and MS-Cu-NPs Synthesized

### 5.3. FTIR Spectrophotometer

FT-IR spectrogram is used to identify and propose a possible interaction that occurs during synthesis of metal nanoparticle by plant extract which is resultant of involving a biomolecules in the reduction. In Figure. 4a&b clearly showed many changing in their spectrum before and after copper nanoparticles fabrication by the *Malva sylvesteris* leaf extract. A strong broad band at 3447 $cm^{-1}$ revealed the presence the O-H vibration of alcohols and phenols and also to the presence of secondary amines N-H of amide. Both 2928 and 2850 $cm^{-1}$ bands attributed to –CH2 and C-H widening mode in pristine extract while in those peaks shifted with decreasing intensity. The bands appearing at 1628 $cm^{-1}$and 1449 $cm^{-1}$ recognized (C-O) stretching in carboxylic group in the formation of oxygen functional groups are shifted in lower and higher filed in the formation of CuNPs to1608 $cm^{-1}$ and 1469 $cm^{-1}$ respectively. Interesting peak appear at 1540 $cm^{-1}$ which attributed to C=C stretching of aromatic ring. The small sharp peak at 1701 $cm^{-1}$in Malva sylvesteris leaf extract is for C=C stretching. 698-1449 $cm^{-1}$ range peaks observed related to alcohols and phenolic groups. The band in the finger print region was detected to be 525 $cm^{-1}$should be a stretching of O-Cu-O[3], [7].

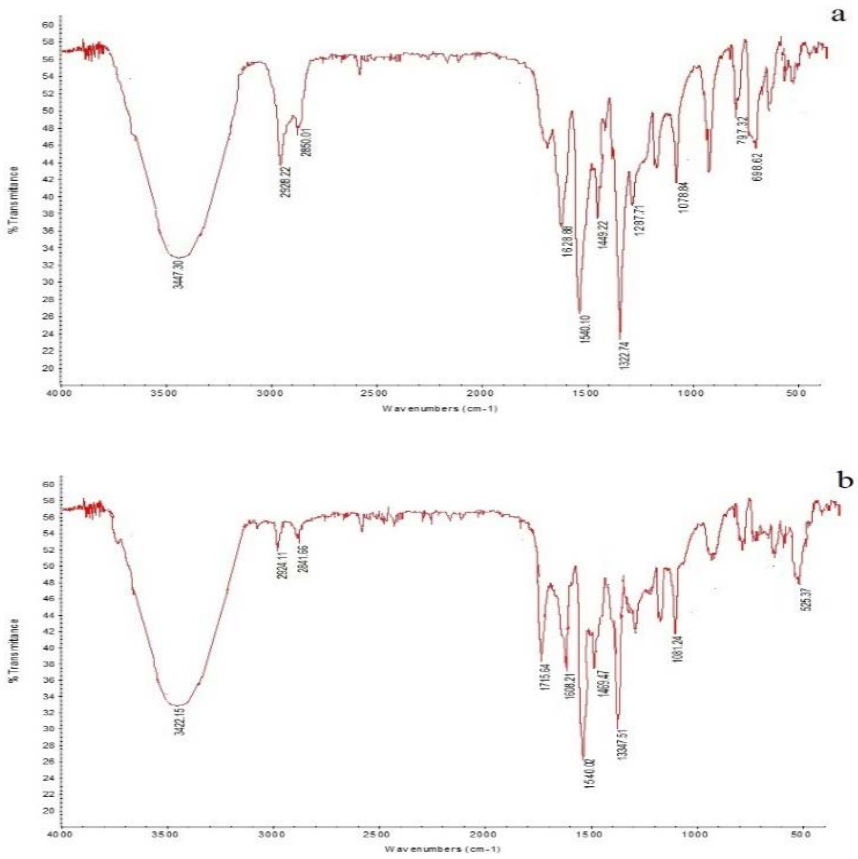


**Figure 4.** FTIR Spectrum of (a) MSLE (b) MS-Cu-NPs synthesized

### 5.4. X-Ray diffraction studies

In Figure. 5 shows particular XRD patterns of shaped MS-Cu-NPs with distinctive peaks of 2θ = 43.23º, 50.12º and 74.12º, with indices of 111, 200 and 220, corresponding to the fcc structure of Copper NPs and consistent with the conventional JCPDS structure (No. 04-0836) data (Table 3). The XRD chart showed no impurity peaks other than Cu showing the elevated purity of the stage. Calculation average size of the MS-Cu-NPs by the formula of Debye – Scherrer as:

$$D= k\lambda/\beta\cos\theta.$$

Where;
D is the particle size (nm).
k is a constant equal to 0.9.
λ is the wave length of X-ray radiation (0.15418 nm),
Full width of the peak at half maximum (FWHM) is β and 2θ is the Bragg angle (degree). The average size of crystallite was discovered to be 14-25 nm in the spectrum.

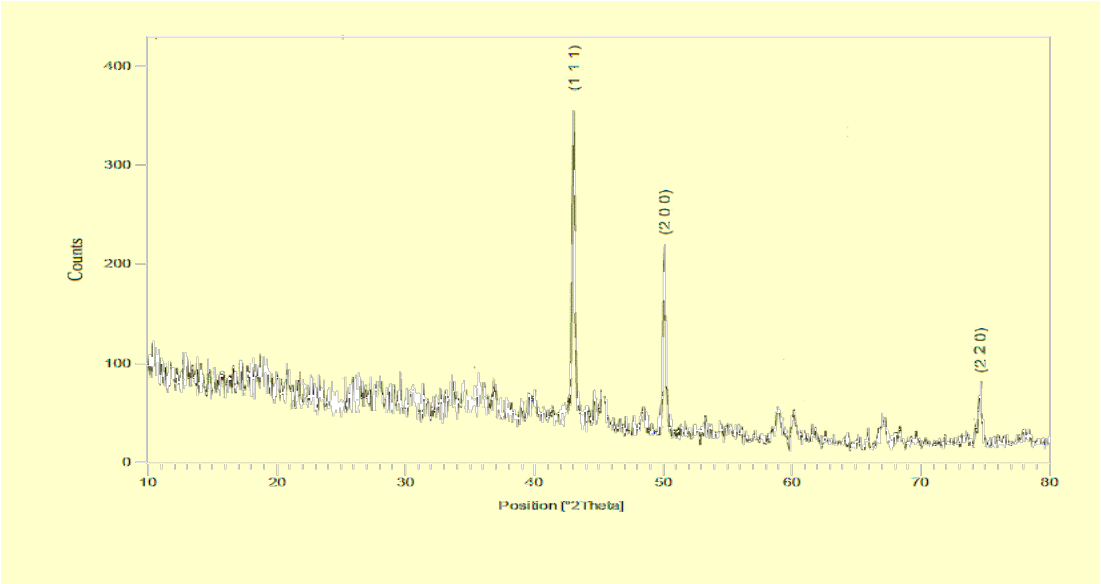


**Figure 5.** XRD spectrum of MS-Copper nanoparticles fabricated by *Malva Sylvesteris* Leaf extract

### 5.5. Antibacterial activity of MS-Cu-NPs

Firstly, MSLE & MS-Cu-NPs have been tested against representative microorganism of bacteria (Gram-negative: *Klebsiella Pseudomonas*; *E. coli*) and (Gram-positive: *Staphy. aureus*; *Clostridium*) at constant volume to show the variation of activity for each one of them (Figure.6), then MIC of MS-Cu-NPs activity was measured against representative microorganism of bacteria (Gram-negative: *Klebsiella Pseudomonas*; *E. coli*) and (Gram-positive: *Staphy. aureus*; *Clostridium*) by micro-dilution broth method and comparing with standard drugs to show the best antibiotic activity at MIC. In agar well-diffusion procedure, the MS-Cu-NPs exhibited important antibacterial effectiveness with the five pathogenic bacteria strains as a function of bio-copper volumes are showed in Figure. 6 were MS-Cu-NPs showed antimicrobial activity against intended pathogenic microorganisms which represented by (Gram-negative: *Klebsiella Pseudomonas*; *E. coli*) and (Gram-positive: *Staphy. aureus*; *Clostridium*) with different degrees, as indicated by the inhibition diameter area, whereas MSLE show smallest antimicrobial activity (Figure. 6). MS-Cu-NPs ' antimicrobial characteristics were evaluated using MIC. Agar – well dilution technique is one of the most frequently used procedures to investigate the MIC of antimicrobial agents; the MIC is described as the smallest antimicrobial agent concentration that prevents a microorganism's noticeable development under specified circumstances. After 24 h of incubation, a heights zones growth have been determined from (16.5-22.5 mm) belong to *E. coli*, *Klebsiella* with (MIC- 12.5, 10, 7.5 mg/ml) and (12-18 mm) for *Pseudomona* while *Staphylococcus*, *Clostridium* showed heights inhibition from (15-20.4 mm) with (MIC- 12.5, 10, 7.5 mg/ml) supplemented by MS-Cu-NPs respectively (Figure. 7). Therefore, MS-Cu-NPs show excellent antibiotic activity comparing with standard drug. In case of assessment of MIC for MS-Cu-NPs, the Gram-negative bacteria (E. coli and Klebsiella) showed greater inhibition and good with (Pseudomonas) compared to the Gram-positive fungi (Staphylococcus aureus; Clostridium) (Figure.7), which may be due to variability in the structure of the cell wall. of Gram-positive bacteria cell wall consists of a dense peptidoglycan layer composed of linear polysaccharide strands crossed by brief peptides, forming a more rigid framework leading to hard penetration of MS-Cu-NPs, while the cell wall has a lower peptidoglycan layer in Gram negative bacteria [8]. Some studies are accessible for the antimicrobial activity mechanism. Anti-bacterial experiments have stated that the Escherichia coli is more efficient bacterial activity and was possibly eliminated with an inhibition area of 35 mm at 20 µl MS-Cu-NPs

(Figure.6) and 31.4 mm at 20 µl MIC-20 mg/ml MS-Cu-NPs (Figure.7). The findings show that NPs mediated the dissipation of cell membrane potential was the likely reason why cell filaments were formed. On the other side, Cu-NPs have been discovered to cause several toxic impacts such as reactive oxygen species generation, lipid peroxidation, protein oxidation, and degradation of DNA in E. coli cells [9]. MS-Cu-NPs ' bactericidal property is primarily due to releasing of the copper cation($Cu^{+}$) and attached to the cell wall of bacteria due to electrostatic interactions. In addition, metal ions not only interact with the surface of the membrane [10], but can also cross the bacteria and eventually bind with DNA molecules and cause helical structure disturbance by networking the nucleic acid strands within and between them [11].

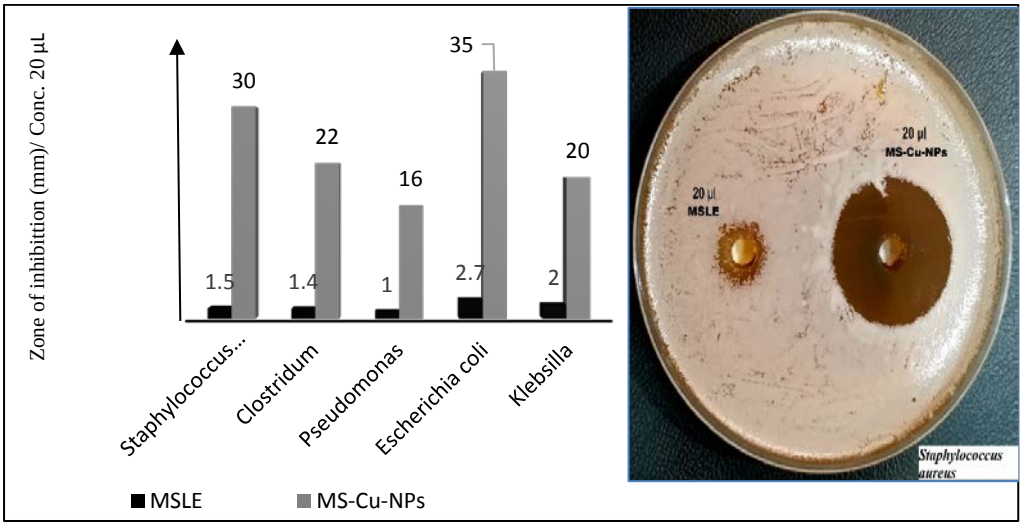


**Figure 6.** Inhibition zone of MSLE and MS-Cu-NPs represent Antiseptic activity against representative microorganism of bacteria (Gram-negative: Klebsiella Pseudomonas; E. coli) and (Gram-positive: Staphy.aureus; Clostridium)

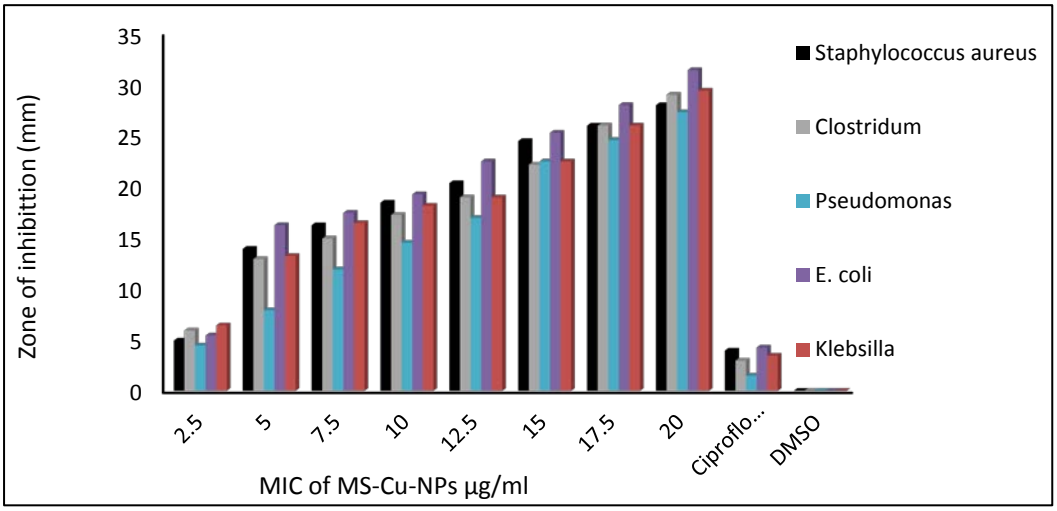


**Figure 7.** MIC (mg/ml) of MS-Cu-NPs against Several bacteria

## 6. CONCLUSION

In this work we first report a clean, cheap cost, environmentally-friendly and suitable method for the synthesis of MS-Cu-NPs utilizing *Malva Sylvesteris* Leaf extract. No chemical reagent or surfactant template in the synthesis of Cu-NPs and even elevated temperature. This technique therefore allows the bioprocess to be environmentally friendly. The extract of *Malva Sylvesteris* identifies by qualitative test and The nanoparticles produced were distinguished by measurements of UV – VIS, FTIR, XRD and had excellent antibacterial activity MIC. A significant prospective benefit of the nanoparticles synthesis technique outlined using Malva Sylvesteris, which is quite stable in solution and is a very precious advantage compared to other biological techniques presently in use. This green reduction technique can be a successful method for preparing other nanoparticles of metals and metal oxide and can be useful in environmental, pharmaceutical, medical and biotechnological applications.

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