*Journal of* Genetic and Environmental Resources Conservation, 2015, 3(3):173-187.
www.jgerc.com

# Preparation of copper oxalate and copper oxide nanoparticles and their antibacterial effect against *P. aeruginosa* and methicillin resistant *S. aureus* from burn and wound infections

Suzan S. Hussain[1], Saadi M.D. Al-Nuzal[2]*and Sarmad F. Al-Qazzaz[3]

[1,3]Biology Department, College of Science, Al-Mustansiriya University and [2]Environmental Research Centre, University of Technology, Baghdad, Iraq.
*Corresponding author: saadidhaher@yahoo.com

## Abstract

Copper oxide and copper oxalate nanoparticles were prepared successfully via soft chemistry route based on the thermal decomposition of oxalic precursors. They were obtained with a well-controlled morphology and particle size. These nanoparticles were characterized by using X-ray diffraction (XRD) pattern analysis, scanning electron microscopy (SEM) and fourier transform infra-red spectroscopy (FT-IR). The antibacterial activity of the obtained copper oxide and copper oxalate nanoparticles against *P. aeruginosa* and MRSA were estimated by using well diffusion method and broth dilution method. The results showed that both types of nanoparticles have the ability to inhibit the growth of these bacterial isolates. The MIC and MBC of copper oxide nanoparticles to *P. aeruginosa* were found to be 1600 and 3200µg/ml respectively, while that for of copper oxalate nanoparticles were 800 and 1600µg/ml respectively. For MRSA the MIC and MBC values of copper oxide nanoparticles were 800 and 1600µg/ml respectively, while that for copper oxalate 200 and 400µg/ml respectively.

Keywords: Copper oxide, Copper oxalate, Nanoparticles, Antibacterial activity, *P. aeruginosa*, *S. aureus*.

## Introduction

The burns and wounds represent a susceptible sites for opportunistic colonization by organism of endogenous and exogenous origin. Bacterial infection in burn and wound patients are common and are difficult to control, and sepsis consequently is common and often fetal. It is responsible for a great deal of morbidity and mortality rate among hospitalized patients (Alharbi and Zayed, 2014).

The rate of nosocomial infections is higher in burn patients due to various factors like nature of burn injury itself, immunocompromised status of the patient, age of patients, extent of injury, duration stay of patient in hospital and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, colonization of the burn wound site and the systemic dissemination of the colonizing organisms (Agnihotri *et al*., 2004; Macedo *et al*., 2005). Currently the common pathogens isolated from burn wound patients are *Pseudomonas aerugenosa*, *Staphylococcus aureus*, β-haemolytic *Streptococci*, *Escherichia coli*, *Klebsiella species* and various coliform bacilli, fungi like *Candida albicans* and *Aspergillus* species are also associated with burn wound infections (Guggenheim *et al*., 2009). Methicillin resistant *Staphylococcus aureus* (MRSA) is a significant pathogen causing both nosocomial and community acquired infections in wound burn patients. *S. aureus* produces a group of virulence factors that causes an array of diseases, ranging from minor localized skin lesions to life-threatening deep tissue damage and systemic infections such as pneumonia, endocarditis and exotoxin syndromes (Matthew, 2012).

Nano-crystalline particles have found tremendous applications in the field of antimicrobials activity and therapeutics, copper oxide nanoparticles (CuO nanoparticles) were effective in killing a range of bacterial pathogens involved in hospital-acquired infections, but a high concentration is required to achieve bactericidal effect. Copper oxide nanoparticles inhibiting cell wall synthesis both of gram-positive and gram-negative bacteria, also it is disrupt DNA structure and biochemical process (Fadhel, 2013; Ren *et al*., 2009). Some methods for the preparation of nanocrystalline CuO have been reported such as the sonochemical method (Kumar *et al*., 2000), sol-gel technique (Eliseev *et al.,* 2000), one-step solid state

reaction method at room temperature (Xu *et al.,*2000)., electrochemical method (Borgohain *et al.,* 2000) and thermal decomposition of precursors (Salavati-Niasari and Davar, 2009). Copper oxide nanoparticles were reported to be effective in killing a range of bacterial pathogens involved in hospital-acquired infections, but a high concentration of nano CuO is required to achieve a bactericidal effect (Ren *et al.*, 2009).

The present study aimed to synthesize copper oxide nanoparticles via soft chemistry route based on the thermal decomposition of oxalic acid salt precursors. Isolation and identification of methicillin resistant *Staphylococcus aureus* and *Pseudomonas aerugenosa* from burns and wounds infections, and their resistance against copper oxide nanoparticles.

## Materials and Methods

Chemicals and biological materials: The following chemicals and biological materials were used in this study: copper nitrate, $Cu(NO_3)_2.3H_2O$ and oxalic acid from Merck, Germany; hydrogen peroxide 3.0% and glycerol from Oxoid, England; barium chloride, $BaCl_2$, BDH, England; hydrochloric acid (HCl), Riedel De Han, Germany; API 20 NE system, biomeruex, France; gram stain Kit, syrbio, Switzerland. Blood agar base, brain heart infusion broth, CHROM agar, mannitol salt agar, muller hinton agar, nutrient agar, pseudomonas agar, and MacConkey agar from hi-media, India; all the antibiotic discs from bioanalyse, Turkey; and pseudomonas agar was prepared according to (Brown, 2005).

Equipments and apparatus: The following equipments and apparatus were used throughout the present study: Fourier Transform Infra-Red spectroscopy (FTIR), shimadzu, Japan; scanning electron microscope, joint stock, Czech republic; Ultrasonic cell crusher, scientz/JY96-IIN, Korea; VITEK2 compact system, biomeruex, France; X- ray diffractometer (XRD-6000), shimadzu, Japan.

Preparation of reagents and solutions: Reagents and solutions were prepared according to the following: Oxidase reagent: (Brown, 2005); catalase reagent, (Forbes *et al*., 2007). Normal saline solution, (McFaddin, 2000), McFarland standard solution, (Benson, 2002). Copper nitrate solution was prepared by dissolving 1.608gm of copper nitrate powder in a mixture of 80ml of 95% ethanol and 20ml of distilled water, oxalic acid solution was prepared by dissolving 0.09gm of solid oxalic acid in 100ml 95% ethanol (Baco-Carles *et al.,*2011).

Samples collection and identification of *P. aerugenosa* and *S. aureus*: Eighty six samples were collected from burn and wound patients from three hospitals of Baghdad governorate: Al-Yarmuk Teaching Hospital; Baghdad Teaching Hospital and Burn Hospital in Medical City. The types and the numbers of clinical samples were distributed as (29) samples from burns and (57) samples from wounds. Samples collected within the period between September 2014 to December 2014, such a way the time between samples collection and bacteriological exam never exceeded 1-2hrs. The swabs were primary cultivated on suitable culture media represent by blood agar and MacConkey agar, then incubated at 37°C for 18-24hrs in aerobic condition. Positive culture samples were re-cultured on further selective media beside performing morphological characteristic, followed by confirmative diagnostic methods (VITEK2 compact system).

*P. aerugenosa* and *S. aureus* were diagnosed according to McFaddin (2000), and all specimens were cultured on MacConkey agar, blood agar, pseudomonas agar as selective media for *P. aeruginosa* and mannitol salt agar as selective media for *S. aureus.* All media were incubated aerobically at 37°C for 24hrs. The bacteria were identified according to their staining ability, shape, color, size, odor, edge, production of pigments and transparency. One isolated colony from selective media was transported to a microscopic slide, fixed then stained with gram stain to determine the cell shape, gram reaction and arrangement (Harley and Prescott, 2002).

Biochemical tests: Oxidase test and catalase tests, (Harley and Prescott, 2002), coagulase test, (Madigan and Martinko, 2005). API 20 NE strip system is a standardized identification system for *Enterobacteriaceae*. Confirmation of the identification of *P. aeruginosa* and *S. aureus* by using VITEK 2 compact system.

Cefoxitin disc diffusion method: Cefoxitin disc diffusion (30µg) was used to detect methicillin resistant *Staphylococcus aureus* (MRSA). (Brown *et al*., 2005).

Antibiotics susceptibility test: Antibiotics susceptibility test were performed by Kirby-Bauer method mentioned in by Morello *et al.* (2006).

Bacterial isolates preservation: Pure bacterial isolates were preserved by three type of preservation, as shown in (Collee *et al*., 1996; Benson, 2002).

Copper oxalate and copper oxide nanoparticles preparation: An alcoholic oxalic acid solution was very slowly added under efficient magnetic stirring to a solution of copper nitrate in alcohol-water at a temperature below 20°C. Addition rate was a such a way it take between 60 to 90min The color of copper nitrate solution was faded during the progress of the addition, and fine suspension of pale blue copper oxalate was observed. The mixture

was left in refrigerator overnight, to allow some of the suspension to settle. The mixture was separated from the mother liquor by centrifugation at 3000 rpm for 60min in 10ml capped plastic tubes. The precipitate was collected in one tube and then washed twice with 95% alcohol, with the aid of the centrifugation at similar rotation speed. After the second washing centrifugation cycle, the particles require more time to sediment. The collected solid (1.53gm) was much less than the theoretical yield, and this could be due to the very small size of resulted copper oxalate. The solid was dried in an oven at 70°C for 5hrs, to give dry solid (1.23gm). It was placed in a small ceramic boat and transferred to a tube furnace, and then heated to 300°C at four steps of heating program. Step one include heating copper oxalate nanoparticles from ambient temperature to 100°C under argon within 60min, step two include heating at 100°C for 60min, step three include heating the sample from 100°C to 300°C within 120min and finally heating at 350°C for 120min.

Due to the high ability of nanoparticles to aggregate during storing, it is recommended to expose their suspensions in distilled water to a sonicator. The probe of the sonicator was inserted in a volume of 100ml of the copper oxide and copper oxalate nanoparticles suspension for 3 × 20min interval at a power of 50% prior to use.

Nanoparticles characterization: The nanoparticle was characterized by X-ray diffraction (XRD) pattern analysis, and molecular analysis of the samples was performed by Fourier Transform Infra-red Spectroscopy (FTIR). The morphology analysis and particle size of samples was carried out by emission scanning electron microscope (SEM).

Determination of antimicrobial activity: Antimicrobial activities of the synthesized copper oxide and copper oxalate nanoparticles were determined against *P. aerugenosa* and MRSA following a modified Kirby Bauer disc diffusion method (Azam *et al*., 2012).

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of copper oxide and copper oxalate nanoparticles were determined by the broth dilution method which conformed to the clinical and laboratory standards institute (CLSI, 2012). The bacterial suspension of *P. aeruginosa* and MRSA were prepared by adjustment of the turbidity of the inoculums to 0.5 McFarland standards ($1.5 \times 10^8$ CFU/ml).

A sequence of four successive dilutions was made by transferring 1.0ml from the above bacteria suspension to 9.0ml NaCl 0.9%. A serial dilution with 5.0ml nutrient broth medium containing nanoparticles at concentration of 200, 400, 800, 1600 and 3200µg/ml was prepared, as well as a positive control without nanoparticles should be done. Each set was inoculated aseptically with 50.0µl of respective diluted bacterial suspension (approximately $10^4$ CFU/ml) including positive control. After incubation at 37°C for 24hrs by using shaking incubator, the bacteria were plated onto solid nutrient agar plates (Azam *et al*., 2012).

## Results and Discussion

Out of a total 86 samples obtained from 29 burns and 57 wounds patients, 75 samples were observed to have an aerobic bacterial growth, while 11, representing, were negative. The absence of the growth in some samples may be related to early entry to the hospital or uninfected patients with aerobic bacteria, as shown in Table (1). Out of the 75 positive growth of aerobic bacteria, 49 (86.0%) were from wound samples, while the positive growth of aerobic bacteria was observed in 26 (89.7%) burn samples out of 29. The colonies were cultured on pseudomonas agar which is selective media to differentiate between *Pseudomonas* genus from other bacteria, this media contains CetriNix supplement for selective isolation of *Pseudomonas* and inhibit other bacteria as well. It enhance the induction of pigments, some isolates of *P. aeruginosa* were able to produce blue-green pigment as pyocyanin and other isolates produce fluorescent pigment as pyovirdin. The confirmatory diagnosis of bacterial isolates were performed by using VITEK2 compact system as an automated microbiological system utilizing growth-based technology.

The results showed that for wound infections, 23 isolates were due to *P. aeruginosa* with the high frequency of 46.9% among 49 positive isolates, while for burn infections, 11 isolates were obtained from 26 positive growth (42.3%). The results obtained for the predominant isolate for burn and wound were in accordance with previous studies (Alharbi and Zayed, 2014; Agnihotri *et al*., 2004; Arslan *et al*., 1999).

The results obtained for the frequency of infection showed that the isolates of *S. aureus* from wound infections represent (24.5%), while in burn infection it was 34.6%. This is close to that reported by Kehinde *et al*. (2004), (26.0%). The identification of methicillin-resistant *Staphylococcus aureus* (MRSA) from wound and burn patients based on use of phenotypic methods including cefoxitin disc diffusion and CHROM agar media for MRSA (Swenson and Tenover, 2005).

Table (1): Numbers and percentage frequencies of burn and wound samples positive and negative for aerobic bacterial infection. Number and percentage of samples with positive aerobic bacterial growth distributed by the source of samples (burns and wounds).

| Sample source | Samples number | Positive aerobic bacterial growth | |
|---|---|---|---|
| | | Number | % |
| Positive samples for aerobic bacteria | | 75 | 87.2 |
| Wound | 57 | 49 | 86.0 |
| Burns | 29 | 26 | 89.7 |
| Negative samples for aerobic bacteria | | 11 | 12.8 |
| Total | 86 | 75 | 87.2 |

The results obtained showed that the isolates of MRSA from wound infections is the predominate isolate with frequency of 75.0%, with 9 isolates of *S. aureus* out of 12, while the rest of MSSA frequency of 25.0%. In burn infections, MRSA isolates were 5 out of 9 with a frequency of 55.6%, while MSSA represent the rest with a value of 44.4%, which is in agreement with the finding of Ekrami *et al*., (2010) of 60.0%. It is worth mentioning that Martineau *et al.* (2000) obtained much higher value of 98%. The high frequency of MRSA can be attributed to many suggested factors, which may include the age of the patients, the high number of MRSA carrier patients, type and volume of burn, immunocompromised patients, and loss of skin barrier. Many studies referred to the abuse of broad spectrum antibiotics prior to the infection and the long residence in hospitals (Shehab El-Din *et al*., 2003).

Thirty four isolates of *P. aeruginosa* from burn and wound patients were tested for antibiotic sensitivity by disc diffusion method (Kirby Bauer Test) according to the recommendation of (CLSI, 2012). The susceptibilities of the isolates towards 10 antibiotic were studied and the antibiotic sensitivity pattern was obtained, for it is a useful guideline for choosing an appropriate antibiotic. These isolates of *P. aeruginosa* showed multidrug resistance (MDR) to more than one antibiotic. The results showed that all isolates were 100.0% resistant to carbenicillin and doxycycline. High resistance to amoxicillin/ clavulanic acid, cefipime, netilmicin and azetreonam showed high resistances of 85.3, 82.4, 76.5 and 61.8% respectively. Moderate resistance were obtained for gentamycin, tobramycin and ciprofloxacin of 35.3, 32.4 and 29.4% respectively. Imipenem was found to be the least resistant antibiotic of 14.7%, as shown in Figure (1) and Table (2).

The development of bacterial resistance towards many antibiotic considered as a great therapeutic problem, and an explanation was presented by Sotto *et al*., (2001) to explain it, which is related to the influence of excessive or inappropriate antibiotic use. *P. aeruginosa* is resistant to many antimicrobial agents and therefore become dominant and important (Brooks *et al*., 2007).

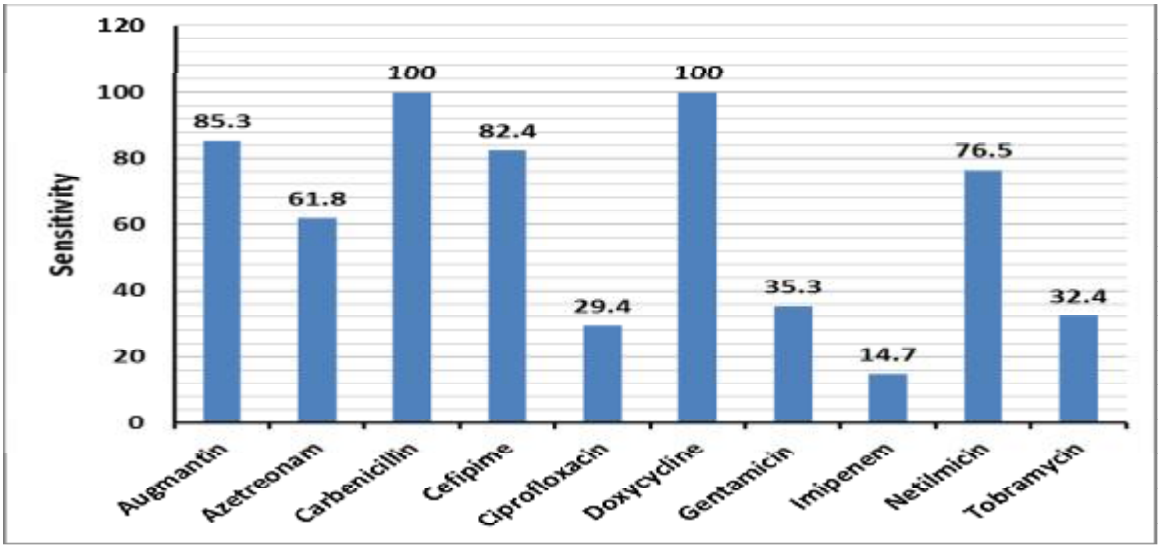


Figure (1): Percentage of resistance for *P. aeruginosa* to antibiotic were used in this study

Table (2): Number and percentage of resistance for *P. aeruginosa* isolates to different antibiotics.

| Antibiotics | Code | *P. aeruginosa* isolates (Number =34) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Resistant | | Intermediate | | Sensitive | |
| | | No. | % | No. | % | No. | % |
| Amoxicillin & Clavulanic acid | AMC | 29 | 85.3 | 1 | 3 | 4 | 11.7 |
| Azetreonam | ATM | 21 | 61.8 | 3 | 8.8 | 10 | 29.4 |
| Carbenicillin | PY | 34 | 100.0 | - | - | - | - |
| Cefipime | FEP | 28 | 82.4 | | | 6 | 17.6 |
| Ciprofloxacin | CIP | 10 | 29.4 | - | - | 24 | 70.6 |
| Doxycycline | DO | 34 | 100.0 | - | - | - | - |
| Gentamicin | CN | 12 | 35.3 | - | - | 22 | 64.7 |
| Imipenem | IPM | 5 | 14.7 | - | - | 29 | 85.3 |
| Netilmicin | NET | 26 | 76.5 | - | - | 8 | 23.5 |
| Tobramycin | TOB | 11 | 32.4 | - | - | 23 | 67.6 |

The results obtained, showed high resistance of *P. aeruginosa* against cefipime 82.4%, this result agreed with both results that were obtained of Bdiwee (2011) and Al-Shwaikh (2006), 98.0%. The three finding were disagreed with gailiene *et al.* (2007), who found the resistance of 20.2%. These difference could be explained to the increase resistance of gram-negative bacteria to cefipime which may be due to repeated exposure to this drug (Livermore *et al*., 2002). The isolates of *P. aeruginosa* was found to have moderate resistance to ciprofloxacin with a value of 29.4%, which is less than that obtained by Eldin *et al*. (2011), of 39%, and much higher than that reported by Abdullah *et al*. (2010) of 6.0%.

Moderate resistance to gentamycin and tobramycin were obtained 35.3 and 32.4% respectively. Al-Shwaikh (2006), reported a close value for the resistance against tobramycin of 26.0%, while Zubair *et al*. (2011), reported the resistance to gentamycin of 40.8%.

The resistance of *P. aeruginosa* to aminoglycosides antibiotic viz. gentamycin and tobramycin, is primarily due to the genetic expression of enzymes responsible for the modification of these antibiotics. There are three specific classes of aminoglycoside-modifying enzyme that have been identified, the N'acetyltransferases, phosphotransferases and adenyltrnsferases (Hirsch *et al*., 2010). *P. aeruginosa* showed high resistance towards amoxicillin/ clavulanic acid (Augmentin), of 85.3%, a close value to that reported by Paul *et al.* (2002) of 90.2%. *P. aeruginosa* showed very low resistance towards Imipenem of 14.7%, which is close to Salimi *et al*. (2009), finding of 15.5%, while Al-Shwaikh (2006), reported ultimate sensitivity (100.0%). *P. aeruginosa* showed complete resistance (100%) towards both of carbenicillin and doxycycline. This result is partially agreed with value reported by Alwan *et al.* (2011) for doxycycline; 73.3%.

Finally, the isolates of *P. aeruginosa* showed low susceptibility of (23.5%) to netilmicin and this partially compatible with Kumar *et al*. (2012), who found only (6.06%) susceptible to this antibiotic and completely compatible with Tanja *et al*. (2013) who reported (29.5%). The high antibiotic resistance of *P. aeruginosa* may be related to many factors such as the widespread use of the broad spectrum antibiotics leading to selective survival advantage of the pathogen (Henrichfreise *et al*., 2007). The close cell-cell contact that permits the bacteria to be more effectively transfer of the plasmids to one another than in the planktonic state. These plasmids can encode for the resistance to several different antimicrobial agents. In addition to the ability of *P. aeruginosa* to form biofilm, which provide a physical protection to the bacteria, hence the biofilm retard the penetration of the antimicrobial agents (Mah and Toole, 2001).

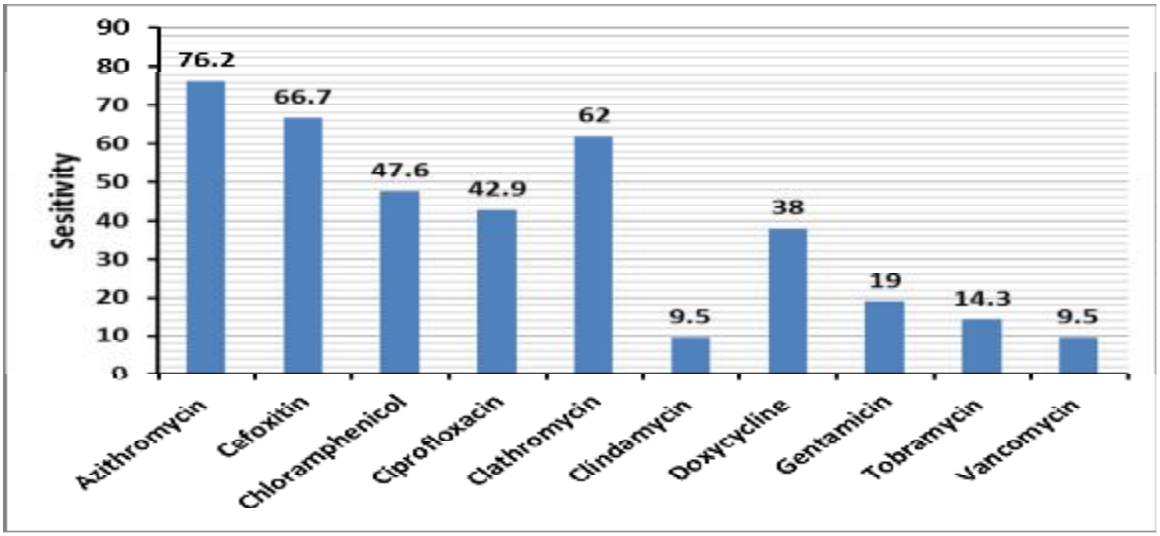


Figure (2): Percentage of resistance for *S. aureus* to antibiotic were used in this study.

According to the recommendation of (CLSI, 2012), twenty one isolates of *S. aureus* isolated from burn and wound patients were tested for antibiotic sensitivity by disc diffusion Method (Kirby Bauer Test). The susceptibilities of the isolates towards 10 antibiotic were studied and the antibiotic sensitivity pattern was for it is a useful guideline for choosing an appropriate antibiotic. The results showed high resistance to azithromycin, cefoxitin, clathromycin with frequencies of 76.2, 66.7 and 62.0% respectively. Moderate resistance to chloramphenicol, ciprofloxacin and doxycycline with frequencies of 47.6, 42.9 and 38.0% respectively. *S. aureus* showed low resistance to clindamycin, vancomycin, tobramycin and gentamycin with frequencies of 9.5, 9.5, 14.3 and 19.0% respectively as shown in Figure (2) and Table (3). The reported values for the resistance of *S. aureus* isolates towards aminoglycosides such as gentamycin and tobramycin antibiotics indicated low resistance of 19.0 and 14.3%, respectively. This finding is completely disagreed with that of Alwan *et al.* (2011) who reported a value of 100.0% for gentamycin. The only floroquinolone used is ciprofloxacin, which showed moderate resistance of 42.9% and this closely agreed with that of Alwan *et al*. (2011), who reported a value 41.6%. Alebachew *et al*. (2012) reported a low resistance of *S. aureus* isolates towards vancomycin and clindamycin 9.7 and 10% respectively. These values are very close to the one obtained in the present study (9.5%).

Table (3): Number and percentage of resistance for *S.aureus* isolates to different antibiotics

| Antibiotics | Code | *S. aureus* isolates (Number =21) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Resistant | | Intermediate | | Sensitive | |
| | | No. | % | No. | % | No. | % |
| Azithromycin | AZM | 16 | 76.2 | - | - | 5 | 23.8 |
| Cefoxitin | FOX | 14 | 66.7 | - | - | 7 | 33.3 |
| Chloramphenicol | C | 10 | 47.6 | - | - | 11 | 52.4 |
| Ciprofloxacin | CIP | 9 | 42.9 | - | - | 12 | 57.1 |
| Clathromycin | CLR | 13 | 62.0 | - | - | 8 | 38.0 |
| Clindamycin | DA | 2 | 9.5 | - | - | 19 | 90.5 |
| Doxycycline | DO | 8 | 38.0 | - | - | 13 | 62.0 |
| Gentamicin | CN | 4 | 19.0 | - | - | 17 | 81.0 |
| Tobramycin | TOB | 3 | 14.3 | - | - | 18 | 85.7 |
| Vancomycin | VA | 2 | 9.5 | - | - | 19 | 90.5 |

Also *S. aureus* showed moderate resistance to doxycycline (38.0%), this accordance with Ahmed *et al*. (2013) who estimated the rate of resistance to this antibiotic was (31.8%). For the chloramphenicol, *S. aureus* isolates were showed moderate percent of resistance (47.6%) and this nearly agreed with Alebachew *et al*. (2012) who reported (51.5%). cefoxitin is a subclass of β-lactams antibiotic which represent a surrogate for the accurate detection of mecA mediated resistance to oxacillin, methicillin and cloxacillin. Results of the study show that (66.7%) of the *S. aureus* isolates were found to be resistant to cefoxitin which also represents methicillin resistant among these isolates, and this percent agreed with Al-Ugaili (2013), who reported (65.6%). Also, the isolates of *S. aureus* showed high resistance (62.0%) to clarithromycin and this compatible with Ahmad *et al*. (2014) who estimated high resistance (59.0%).

In an attempt to prepare copper compounds nanomaterials, the strategy of using sparingly soluble oxalates precursors formation from dilute solution followed by thermal decomposition of the dry product was adopted. The first part of the preparation consist of the formation of insoluble copper oxalate from diluted aqueous alcoholic solution, followed by centrifugation, drying and thermal decomposition. The following chemical equations represent the decomposition process:

$$Cu(NO_3)_2 + H_2C_2O_4 \longrightarrow CuC_2O_4\downarrow + 2HNO_3 \quad \ldots\ldots 1$$

$$CuC_2O_4 \xrightarrow{75\text{-}263^\circ C} CuCO_3 + CO\uparrow \quad \ldots\ldots 2$$

$$CuCO_3 \xrightarrow{263\text{-}325^\circ C} CuO + CO_2\uparrow \quad \ldots\ldots 3$$

Copper metal nanoparticles are mainly attractive due to their catalytic optical and electrical properties (Athanassiou *et al*., 2006). Unfortunately they are unstable in air due to oxidation although they can be conserved in an inert medium (Jana *et al*., 2000). However, due to the high reactivity of nanoparticles especially concerning oxidation, pure copper metal is not easy to stabilize. Although, Baco-Carles *et al*. (2011) claimed the synthesis of nanoparticles of copper metal via a soft chemistry route. However, the process required to prepare metallic copper nanoparticles should be well closed system to exclude oxygen and water contact with the product. Such system need a prolonged preparation, as well as the required safety precaution for handling hydrogen gas as a reducing agent at a temperature exceeding 350ºC for the copper metal.

Following the above mentioned procedure with modification, copper oxalates precipitated from water-alcohol medium is expected to give submicron size of the precursor grains. Moreover it decompose at a temperature above 200°C to prevent dramatic sintering of the metallic particles formed, and their decomposition gives water and carbon compounds, which do not lead to real human health problems. Referring to an extensive study performed by Feng Qi *et al*., (2014), who studied the thermal decomposition of copper(II) oxalate, $CuC_2O_4$ obtained by aging of CuO and oxalic acid $H_2C_2O_4 \cdot 2H_2O$ at 45°C without the use of solvent. The decomposition thermogram showed that the main weight loss in copper oxalate took place in two stage; the first stage is within a temperature range of 75-263°C, and the second stage 263-325ºC. The first decomposition stand for equation-2, and the second major decomposition stand for the equation-3. He calculated CuO residue to be 50.2%, and the calculated water loss to be 5.6%. According to this calculation the weight loss was 55.8%.

Copper oxalate was prepared by following the procedure, mentioned in experimental part and product was obtained as a pale blue powder, sparingly soluble in water and ethanol and represented by the formula shown in Figure (3). Its formula consist of one copper cation attached to one oxalate anion.


Figure (3): Copper oxalate formula, in which one molecule of copper cation attached to one oxalate anion.

For the preparation of copper oxide nanoparticles, an amount of copper oxalate was decomposed under argon atmosphere in a tube furnace with programmed heating up to 350°C. This programmed heating is based on the behavior of copper oxalate described by Feng Qi *et al*., (2014). Complete conversion of the oxalate took place within this heating regime. The product was characterized by the same analytical techniques used for copper oxalate. Structural and morphological properties of copper oxalate $Cu_2C_2O_4$ and copper oxide CuO nanoparticles accomplished by using, X-ray Diffraction (XRD), scanning electron microscopy (SEM) and fourier transform-infrared spectroscopy (FT-IR) at Nanotechnology and Advanced Materials Center, The University of Technology.

The recorded FT-IR spectrum of CuO nanoparticles in KBr disc in the range 400 -4000$cm^{-1}$ are shown in Figure (4). It showed only broad band with many tiny top peaks at 478.99, 522.71, 534.28 and 570.93$cm^{-1}$, which belong to the vibration frequency of Cu-O nanoparticles. Confirming the formation of CuO nanoparticles. The weak band around 2300 may attributed to the vibration of atmospheric $CO_2$.

The recorded FT-IR spectrum of $CuC_2O_4$ nanoparticles in KBr disc is shown in Figure (5). It showed broad band within the range 450 - 500$cm^{-1}$ which stand for the vibration of Cu-O bond. Another set of absorption peaks at 1624.06, 1514, 1477.47, 1406.11, 1398.39 and 956$cm^{-1}$, which are belong to the vibration of the oxalate structure -O-C(O)-C(O)-O-, confirming the formation of calcium oxalate $CuC_2O_4$ nanoparticles.

XRD spectrum of copper oxide nanoparticles (Figure 6), showed peaks at 33.0°, 36.0°, 39.0°, 49.0°, 53.0°, 58.0°, 62.0°, 66.0° and 67.0° and all of them were characteristic signal for this compound. XRD spectrum of copper oxalate nanoparticles (Figure 7), showed a set of broad peaks at 23°, 26° and 29° due to the presence of oxalate carbon atom, and another set of broad peak at 33.0°, 36.0°, 39.0° and 49.0° and all of them were characteristic signal for the presence of copper atom (Feng Qi *et al*., 2014).

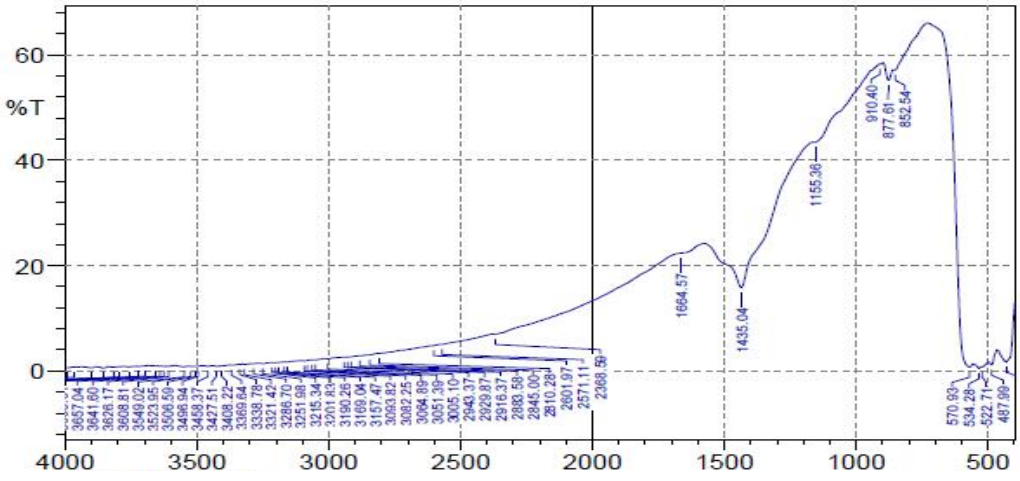


Figure (4): The FT-IR absorption spectrum of copper oxide.

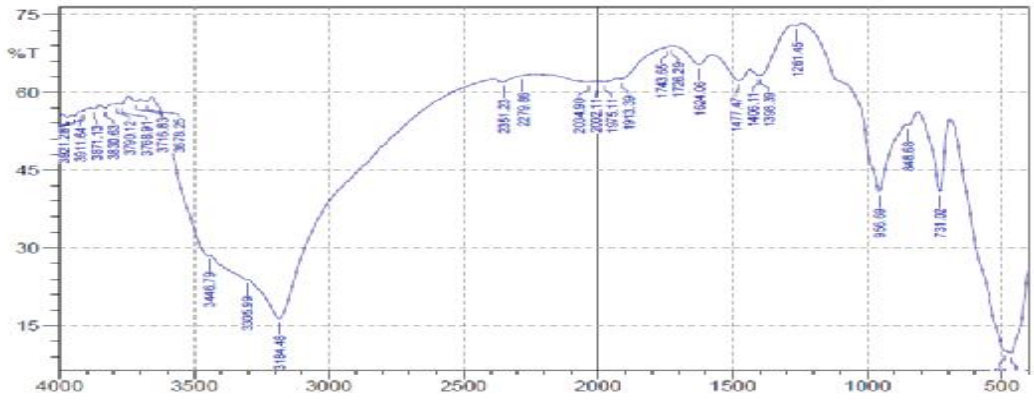


Figure (5): The FT-IR absorption spectrum of copper oxalate.

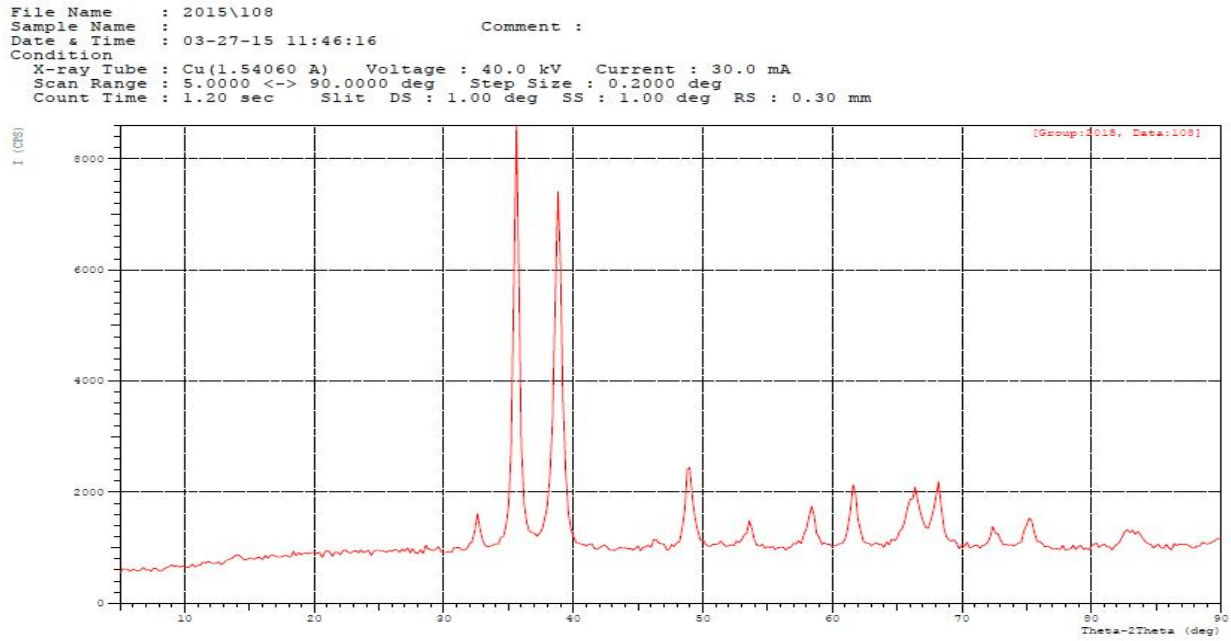


Figure (6): The X-ray diffraction spectrum of copper oxide.

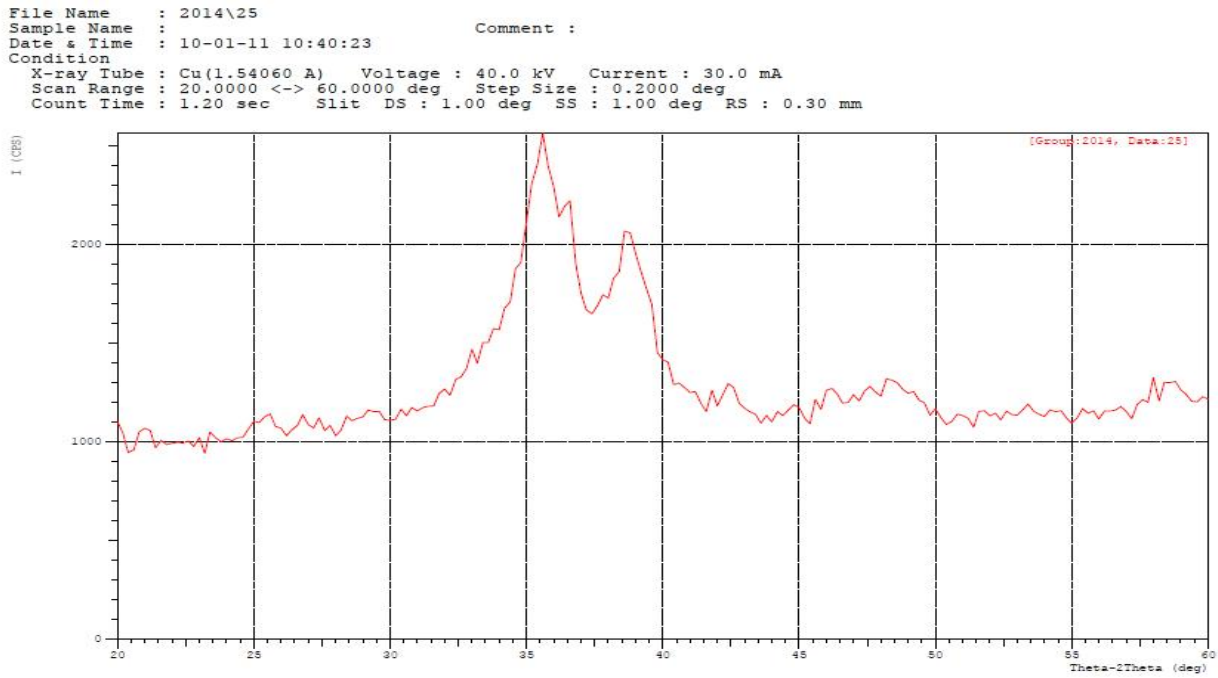


Figure (7): The X-ray diffraction spectrum of copper oxalate.

Scanning Electron Microscope (SEM) image of copper oxalates precipitated from water-alcohol medium following the procedure mentioned in the experimental part, showed that the precipitate particles were composed of aggregated very small particles of ~ 50 -100nm, as shown in Figure (8). This is an unexpected observation; nanoparticles were formed by precipitation from solution, because it was has been mentioned to obtain submicron size of the grains (Baco-Carles *et al*., 2011). The image clearly showed the aggregation of the grains of high surface area.

SEM image of copper oxide obtained by programmed thermal decomposition, showed that its particles were composed of aggregated very small particles of ~ 50nm, as shown in Figure (9). The image clearly showed the aggregation of the grains of high surface area.

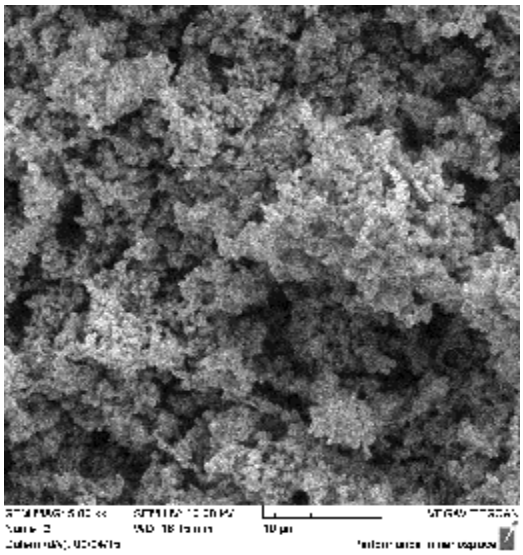

Figure (8): The Scanning Electron microscope image of copper oxalate.

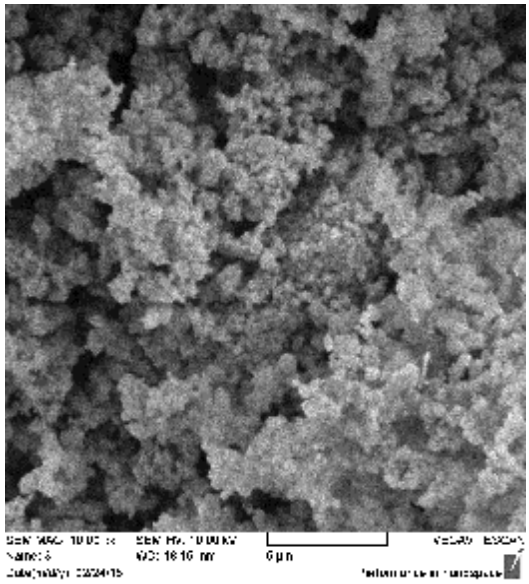

Figure (9): The scanning electron microscope image of copper oxide.

The antibacterial activity of copper oxide and copper oxalate nanoparticles were tested against two pathogenic bacterial strains of *P. aeruginosa* and MRSA. Two common methods were used for this purpose, the first method was agar well diffusion method, and the other was the broth dilution method (Azam *et al*., 2012). Five concentrations of copper oxide and copper oxalate nanoparticles (200, 400, 800, 1600 and 3200µg/ml), were used to determine the antibacterial effect by following the procedure mentioned in experimental part. The following observation can be drawn from the result of the experiment, as shown in Figure (10):

1. The results showed that both types of nanoparticles do not have antibacterial activity against *P. aeruginosa* at a concentration of 200µg/ml while, both types of nanoparticles do not have antibacterial activity against MRSA at concentrations of 200 and 400µg/ml.
2. Copper oxide nanoparticles start to show antibacterial effect against *P. aeruginosa* at a concentration of 400, 800, 1600 and 3200µg/ml, with inhibition zone of 10, 12, 15 and 20mm, respectively. As far as we know this is the first report on the antibacterial activity of copper oxalate nanoparticles against *P. aeruginosa*.
3. Copper oxalate nanoparticles start to show a higher antibacterial effect against *P. aeruginosa* at a concentration of 400, 800, 1600 and 3200µg/ml, with inhibition zone of 18, 20, 25 and 35mm, respectively. Although the particle size of copper oxalate is higher than that of copper oxide, but it diffusion through the agar media is almost twice that of copper oxide. Probably there more ionic property in copper oxalate than that of copper oxide, as well as the toxic contribution of the oxalate ions.
4. For MRSA, the copper oxide nanoparticles which given inhibition zone about 8, 15 and 20mm to the concentrations 800, 1600 and 3200µg/ml, respectively. This data reveal that higher concentration of copper oxide is required to inhibit the growth of MRSA. Similar observation was found for copper oxalate nanoparticles with inhibition zone of 10, 13 and 17mm for the same observation. The assumption of easier diffusion of copper oxalate is no longer useful, probably due to the unique resistance of MRSA strains. This is the first report on the antibacterial activity of copper oxalate nanoparticles against MRSA.

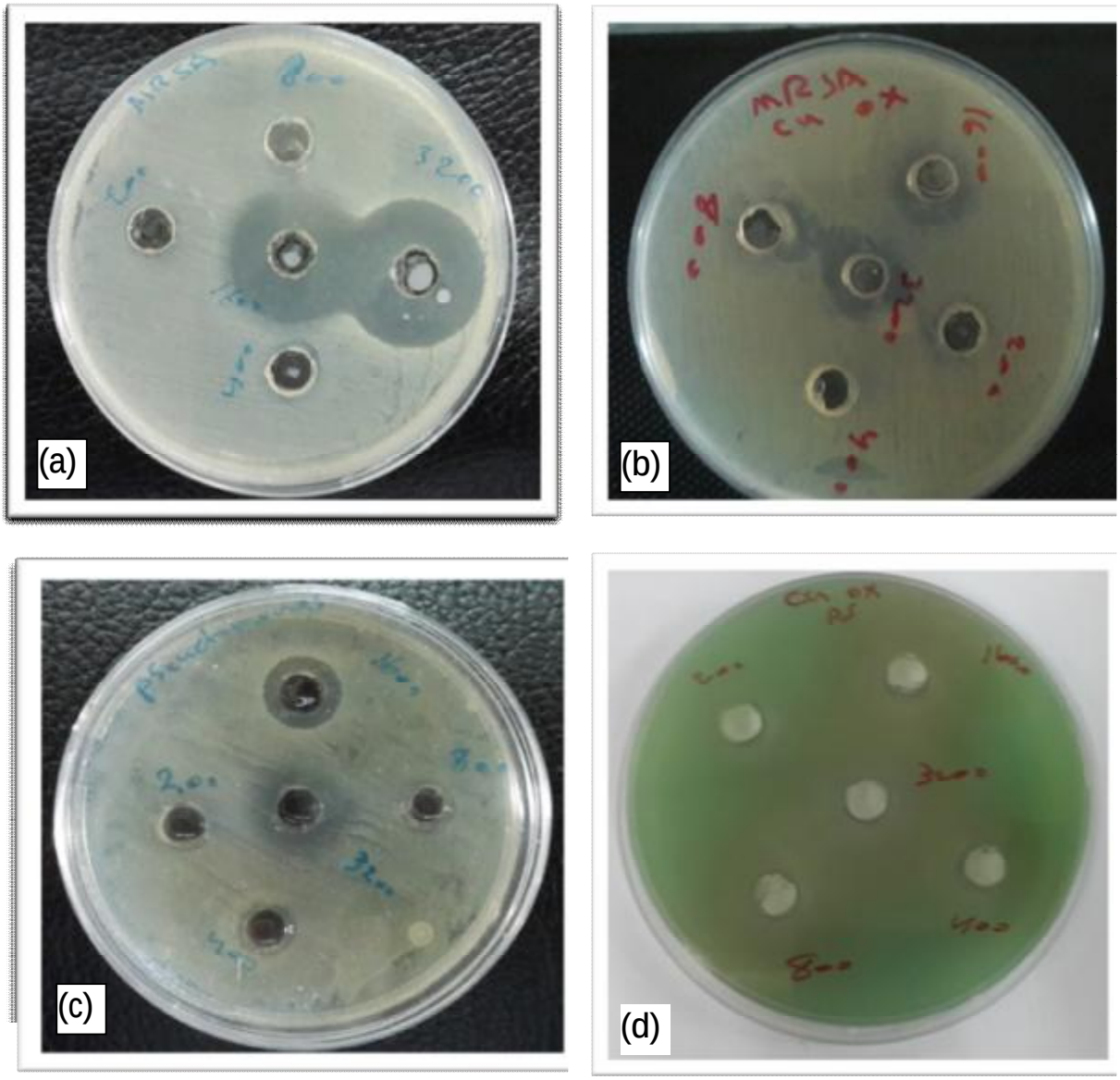


Figure (10): The antibacterial activity (a) copper oxide nanoparticles against MRSA, (b) copper oxalate nanoparticles against MRSA, (c) copper oxide nanoparticles against *P. aeruginosa* and (d) copper oxalate nanoparticles against *P. aeruginosa*.

The results were obtained from this method revealed that the antibacterial activity of both types of nanoparticles against *P. aeruginosa* and MRSA increase directly proportional with increase in their concentrations as shown in Figures (10 and 11). The lowest concentration inhibiting the bacterial growth was defined as the minimal inhibitory concentration (MIC), in contrast the minimal concentration which completely inhibited the bacterial growth was defined as the Minimum Bactericidal Concentration (MBC) (Azam *et al.,* 2012). Five concentrations of copper oxide and copper oxalate nanoparticles (200, 400, 800, 1600 and 3200µg/ml) and a control solution, were used to determine the antibacterial effect, MIC, and MBC. The effect of both nanoparticles were determined by following Broth dilution method, as mentioned in the experimental part.

The methodology and procedure of Clinical and Laboratory Standards Institute (CLSI, 2012) was followed through a 5.0ml serial dilutions of nutrient broth medium containing nanoparticles, with the above mentioned concentration. The following observations can be drawn from the result of this experiment:

1. The MIC and MBC values of copper oxide nanoparticles against *P. aeruginosa* were at concentration of 1600 and 3200µg/ml respectively.

2. The MIC and MBC value of copper oxalate nanoparticles against *P. aeruginosa* were at concentration 800 and 1600µg/ml, respectively.
3. The MIC and MBC value of copper oxide nanoparticles against MRSA were found at concentrations 800 and 1600µg/ml, respectively.
4. The MIC and MBC value of copper oxalate against MRSA were at concentrations 200 and 400µg/ml respectively.

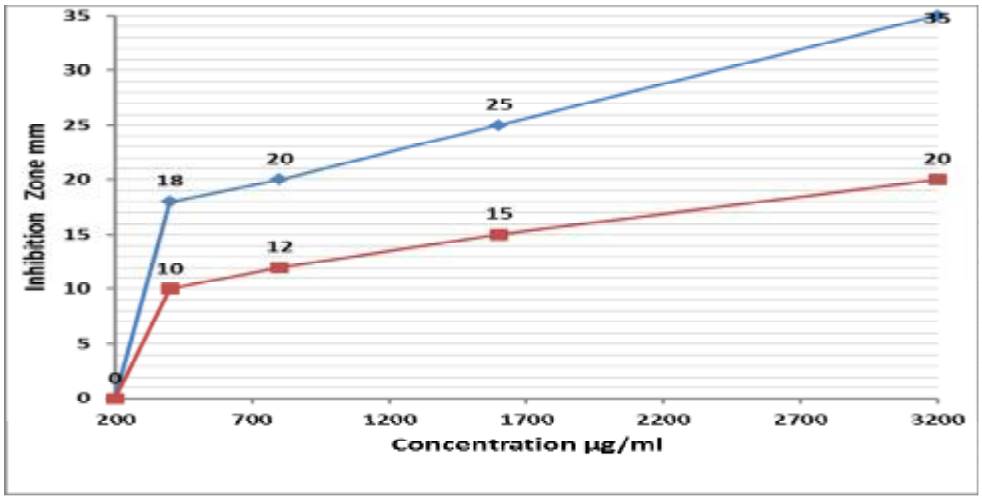


Figure (11): The antibacterial effect of different concentration of copper oxide (blue line) and copper oxalate (red line) nanoparticles against *P. aeruginosa*.

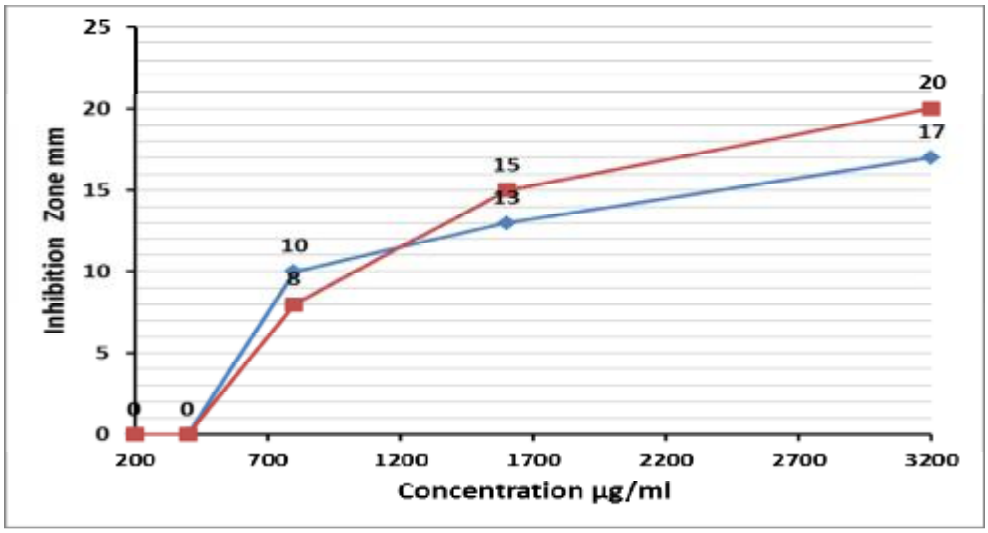


Figure (12): The antibacterial effect of different concentration of copper oxide (blue line) and copper oxalate (red line) nanoparticles against MRSA.

Table (4): The MIC and MBC values of copper oxide and copper oxalate nanoparticles against *P. aeruginosa* and MRSA.

| Nanoparticle | Microorganism | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 200 | 400 | 800 | 1600 | 3200 |
| Copper oxide | *P. aeruginosa* | NK | NK | NK | MIC | MBC |
| | MRSA | NK | NK | MIC | MBC | K |
| Copper oxalate | *P. aeruginosa* | NK | NK | MIC | MBC | K |
| | MRSA | MIC | MBC | K | K | K |

*MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; NK: no killing; and K: killing.

The above results and the observations drawn from it, they are in agreement with other worker results. High concentrations of nanoparticles have been used to achieve the antibacterial activity against MRSA isolates according to Ren *et al*. (2009), who reported a high concentration of copper oxide nanoparticles were required to achieve a bactericidal effect, with MBC of 5000µg/ml.

It is clear from Table (4), the *P. aeruginosa* seemed to be more resistant to both types of nanoparticles than MRSA, and this observation is in agreement with earlier studies by Zarrindokht and Chehrazi (2011). It was earlier reported that the interaction between gram-positive bacteria and nanoparticles was stronger than that of Gram-negative bacteria and this is due to several reasons including the difference in cell walls between them, the cell wall of *P. aeruginosa* which consists of lipids, proteins and lipopolysaccharides (LPS), provides effective protection against biocides. However, the cell wall of gram-positive bacteria such as *S. aureus* does not consist of LPS (Speranza *et al*., 2004). It has been suggested that the reduced amount of negatively charged peptidoglycans makes gram-negative bacteria such as *P. aeruginosa* less susceptible to such positively charged antimicrobials. The physiology, metabolism and degree of contact of organisms with nanoparticles will influence the resistivity of the various types of bacteria (Azam *et al*., 2012).

Generally, Nanoparticles show efficient antibacterial property due to their extremely large surface area, which provides better contact with microorganisms and copper ions released subsequently, may bind with DNA molecules and lead to disorder of the helical structure by cross-linking within and between the nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical processes then cell death (Abboud *et al.,* 2014). Broadly, interactions between the negative charges of microorganisms and the positive charges of nanoparticles produces an electromagnetic in attraction between the microbe and effective levels of active nanoparticles. Such interactions lead to oxidation of surface molecules of microbes resulting in their death (Azam *et al.,* 2012).

Copper oxide and copper oxalate nanoparticles inhibit the growth of both gram-negative and Gram-positive bacteria. It is worth mentioning that there is no previous work on copper oxalate nanoparticles antibacterial activity against *P. aeruginosa* and MRSA.

## References


Abboud, Y.; Saffaj, T.; Chagraoui, A.; El-Bouari, A.; Brouzi, K.; Tanane,O. and Ihssane, B. 2014. "Biosynthesis, characterization and antimicrobial activity of copper oxide nanoparticles (CONPs) produced using brown alga extract (*Bifurcariabifurcata*). Appl. Nanoscie., 4:571-576.

Abdulluh, R.M.; Samaan, S.F. and Al-Shwaikh, A.M. 2010. Study the Effect of antibiotic combination of beta-lactam and amino glycoside with another group of antibiotics and their synergism effect. J. Arab Board Health Specializ., 11(1): 324-355.

Agnihotri, N.; Gupta, V. and Joshi, R.M. 2004. Aerobic bacterial isolates from burn wound infections and their antibiograms: a five-year study. Burns 30: 241-243.

Ahmed, B.; Khan, F.; Ahmed, J.; Cha, B.; Shin, M. and Basher, S.H. 2014. Antibiotic resistance Pattern and molecular Epidemiology of methicillin resistant *Staphylococcus aureus* colonization in Burns unit of a Tertiary care Hospital in Peshawar, Pakistan. Trop. J. pharm. Res., 13(12): 2091-2099.

Ahmed, J.; Ali, A.; Hussain, M. and Ahmed, H. 2013. Antimicrobial Susceptibility Pattern of bacteria isolated from Burn wounds in a tertiary care Hospital in Pakistan. Afr. J. Microbiol. Res., 7(28): 3627-3631.

Alebachew, T.; Yismaw, G.; Derabe, A. and Sisay, Z. 2012. *Staphylococcus aureus* burn wound infection among patients attending Yekatit 12 hospital burn unit, AddisAbaba, Ethiopia. J. Health. Sci., 22(3): 209-213.

Alharbi, S.A. and Zayed, M.E. 2014. Antibacterial susceptibility of bacteria isolated from burns and wounds of cancer patients. J. Saudi Chem. Socie., 18: 3-11.

Al-Shwaikh, R.M. 2006. Production and characterization of protease from *Pseudomonas aeruginosa* isolated from some clinical cases and its relation with some antibiotic agents. Ph.D. Thesis Biology, Microbiology, College of Science, Al-Mustansiriya University.

Al-Ugaili, D.N. 2013. Bacteriological and genetic studies on oxacillin resistant *Staphylococcus aureus* isolated from hospitals in Baghdad city. Ph.D. College of Science, Al- Nahrain University.

Alwan, M.J.; Lafta, I.J. and Hamzah, A.M. 2011. Bacterial isolation from burn wound infections and studying their antimicrobial susceptibility. Kufa J. Vet. Med. Sci., 2(1): 121-131.

Arslan, E.; Dalay, C.; Yavus, M.; Gocenler, L. and Acartuk, S. 1999. Gram negative bacterial Surveillance in burn patients. Annals of Burn and Fire Disasters. Vol. XII, No. 2.

Athanassiou, E.K.; Grass, R.N. and Stark, W.J. 2006. Large-scale production of carbon-coated copper nanoparticles for sensor applications. Nanotechnol., 17(6): 1668-1673.

Azam, A.; Ahmed, A.; Oves, M.; Khan, M.; Habib, S.S., and Memic, A. 2012. Size-dependent antimicrobial properties of CuO nanoparticles against gram-positive and negative bacterial strains. Inter. J. Nanomed., 7: 6003–6009.

Baco-Carles, V.; Datas, L. and Tailhades, P. 2011. Copper Nanoparticles Prepared from Oxalic Precursors., Inter. Scholarly Res. Network, 2011: 1-7.

Bdiwee, S.G. 2011. Assessment of the resistance of the *Mycobacterium tuberculosis* to antibiotics and investigating the Associated Bacteria in the Patients. M.Sc. Thesis in Microbiology, College of Science for Women, University of Baghdad.

Benson, J.H. 2002. Microbiological Applications, Laboratory Manual in General Microbiology. 8th ed., The McGraw-Hill Higher Education, New York.

Borgohain, K.; Singh, J.B.; Rama, M.V.; Shripathi, T. and Mahamuni, S. 2000. Quantum size effects in CuO nanoparticles. Phys. Rev., 61(16): 11093-11096.

Brooks, F.G.; Carroll, K.C.; Butel, S.J. and Morse, A.S., 2007. Medical Microbiology, Jawetz, E., Melnic, J.L. and Adelberg, E.A., 24th ed. McGraw-Hill. New York.

Brown, A.E., 2005. Benson`s Microbiological Applications: Laboratory Manuals in General Microbiology. 9th ed., McGraw-Hill Company, USA.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance Standards for Antimicrobial Susceptibility Testing; Approved Standard. 11th ed., M02-A11, Vol. 32, No. 1.

Collee, J. G.; Fraser, A.G.; Mormion, B.P. and Simmons, A. 1996. Mackie and McCartney Practical Medical Microbiology. 14th ed., Churchill Livingstone, New York, 151-78pp.

Ekrami, A.; Samarbafzadeh, A.; Alavi, M.; Kalantar, A. and Hamzeloi, F. 2010. Prevalence of methicillin-resistant *Staphylococcus* species isolated from burn patients in a burn center, Ahvaz, Iran. Jundishapur J. Microbiol., 3(2): 84-91.

Eldin, E.S.; Abdallah, S.; El-Dein, A.; Shawki, M. and Eldin, A.G. 2011. Comparison between outer membrane protein profile of fluoroquinolones sensitive and resistant *Pseudomonas aeruginosa* isolated from Egyptian patients. J. American Sci., 7.

Eliseev, A.A.; Lukashin, A.V.; Vertegel, A.A.; Heifets, L.I.; Zhirov, A.I. and Tretyakov, Y.D., 2000. Complexes of Cu(II) with polyvinyl alcohol as precursors for the preparation of $CuO/SiO_2$ nano composites. Materials Res. Innov., 3(5): 308–312.

Feng Qi, R.; Stein, S. and Friscic, T. 2014. Mimicking minerals neogenesis for the clean synthesis of metal-organic materials from mineral feedstock: coordination polymers MOFs and metal oxide separation. Green chem., 16: 121-132.

Forbes, B.A.; Sahm, D.F. and Weissfeld, A.S., 2007. Diagnostic Microbiology. 11th ed., Mosby, Inc., Baltimore, USA, 302-309pp.

Gailiene, G.; Pavilonis, A.; and Areiviene, L. 2007. The peculiarities of *Pseudomonas aeruginosa* resistance to antibiotics and prevalence of serogroups. Medicina., 43(1): 36-42.

Harley, J.P. and Prescott, L.M. 2002. Laboratory Exercises in Microbiology. 5th ed., The McGraw-Hill Companies Inc., New York, 292-293pp.

Henrichfreis, B.; Wiegand, I.; Pfister, W. and Wiede, B. 2007. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hyper mutation. Antimicrob. Agents. Chemother., 51(11): 4062–4070.

Hirch, E.B.; Tam, V.H.; Rogers, C.A.; Chang, K.; Weston, J.S. ; Cairo, J. and Garey, K.W., 2010. Impact multidrug resistant *Pseudomonas aeruginosa* infection on patients outcomes. Expert. Rev. pharma. outcomes Res.,10(4): 441-451.

Jana, N.R.; Wang, Z.L.; Sau, T.K. and Pal, T. 2000. Seed-mediated growth method to prepare cubic copper nanoparticles. Langmuir, 33(10): 5740-5745.

Kehinde, A.O.; Ademola, S.A.; Okesola, A.O.; Oluwatosin, O.M. and Bakare, R.A. 2004, "Patients of bacterial pathogens in burn wound infections in Ibadan, Nigeria. Ann. Burn Fire Disas., Vol. XVII, No. 1.

Kumar, R.V.; Diamant, Y. and Gedanken, A. 2000. Sonochemical synthesis and characterization of nanometer-size transition metal oxides from metal acetates. Chem. Mater., 12(8): 2301-2305.

Livermore, D.M. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa* our worst nightmare. J. Clin. Infect. Dis., 346: 34-640.

Macedo, J.L.S. and Santos, J.B. 2005. Bacterial and fungal colonization of burn wounds. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, 100(5): 535-539.

Madigan, M.M. and Martinko, J. 2003. Brock Biology of Microorganisms. 10th ed., Prentice Hall, Upper Saddle River, NJ, USA.

Mah, T. and O'Toole, G. 2001. Mechanisms of Biofilm Resistance to Antimicrobial Agents. J. Trends in Microbiol., 9: 34-39.

Martineau, F.; Picard, F.J.; Lansac, N.; Menard, C.; Roy, P.H.; Ouellette, M. and Bergeron, M. G. 2000. Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother., 44: 231-238.

Matthew, J.T. 2012. Synthesis of the accessory gene regulator auto-inducing peptide in *Staphylococcus aureus*. Ph. D Thesis, The Graduate College, University of Iowa, USA.

McFaddin, J.F. 2000. Biochemical Test For Identification of Medical Bacteria. 3rd ed., The Williams and Wilkins. Baltimore, USA.

Merlin G.; Reinhard, Z.; Alexander E.; Handschin, A.G.; Mehmet, A.A. and Pietro, G. 2009. Changes in bacterial isolates from burn wounds and their antibiograms: A 20-year study (1986–2005). Burns, 5(4): 553–560.

Paul, S.R.; Bezbarauh, M.K.; Roy, A.C and Ghosh, M. 2002. Multiple antibiotic resistance (MAR) index and its reversion in *Pseudomonas aeruginosa*. Lett. Appl. Microbiol., 24: 169-171.

Rawan A.A.; Suzan S.H. and Ali R.L. 2014. Antibacterial and antibiofilm activity of zinc oxide nanoparticles on *Pseudomonas aeruginosa* isolates from diabetic foot infections grade II. Al-Mustansiriyah J. Pharmaceut. Sci., 14(2): 15-21.

Ren, G.; Hu, D.; Cheng, E.W.; Vargas-Reus, M.A.; Reip, P. and Allaker, R.P. 2009. Characterization of copper oxide nanoparticles for antimicrobial applications. Intern. J. Antimicrob. Agents., 33: 587–590.

Salavati-Niasari, M. and Davar, F. 2009. Synthesis of copper and copper (I) oxide nanoparticles by thermal decomposition of a new precursor. Mater Lett., 63(3-4): 441-443.

Salimi, H.; Owlia, P.; Yakhchali, B. and Lari, A.R. 2009. Drug Susceptibility and Molecular Epidemiology *Pseudomonas aeruginosa* Isolated in a Burn Unit. Am. J. Infect. Dis., 5(4): 308-313.

Sotto, A.; De Boever, C.M.; Fabbro-Peray, P.; Gouby, A.; Sirot, D. and Joudan, J. 2001. Risk factors for antibiotic resistant *Escherichia coli* isolated from hospitalized patients with urinary tract infection: a Prospective study. J. Clinical Microbiol., 39: 438-444.

Speranza, G.; Gottardi, G.; Pedrazoli, C.; Lunelli, L. and Carli, E. 2004. Role of chemical interaction in bacterial adhesion to polymer surfaces. Biochemicals, 25: 2029-2037.

Swenson, J.M. and Tenover, F.C. 2005. Cefoxitin disc Study group result of disc diffusion testing with cefoxitin correlates with presence of mecA in *Staphylococcus* sp. J. Clin. Microbiol., 43: 3818-3823.

Tanja, N.; Chari, P.S.; Sigh, M.; Sigh, G.; Biswal, M. and Sharma, M. 2013. Evolution of bacterial flora in burn wounds: Key role of environmental disinfection in control of infection. Int. J. Burn Trauma, 3(2): 102-107.

Zarrindokht, E.K. and Cheharzi, P. 2011. Antibacterial activity of ZnO nanoparticles on Gram positive and Gram negative bacteria. Afr. J. Microbiol. Res., 5: 1368-1377.

Zubair, M.I.; Malik, A.I.; Ahmad, J.Z.; Rizvi, M.I.; Farooqui, K.J. and Rizvi, M.W. 2011. A Study of Biofilm Production by Gram-negative organisms isolated from diabetic foot ulcer patients. J. Biol. Med., 3(2): 147-157.