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Synthesis of Silver Nanoparticles and Incorporation with Certain Antibiotic Using Gamma Irradiation

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Authors' contributions

This work was carried out in collaboration between all authors. They designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The study was explaining that silver nanoparticles (AgNPs) were synthesized biologically by *Bacillus megaterium* culture supernatant (as reducing and stabilizing agents) by the optimization of medium components for nitrate reductase production to enhance the synthesis of AgNPs. And use of gamma irradiation for the synthesis and incorporation of AgNPs with selected antibiotics at distinct dose.
Place and Duration of Study: The study was carried out in 2012 in the Department of Drug Radiation Research, Egyptian Atomic Energy Authority.
Methodology: The optimized conditions for AgNPs formation by *B. megaterium* culture supernatant were as follows; media containing: (%) yeast extract: 0.15, peptone: 0.25, KNO_3:0.1 temp: 30ºC and incubation period 24 h with maximum nitrate reductase activity of (680.89U/ml). Physical synthesis of AgNPs and incorporation with antibiotics such as (Sodium Cefotaxime, Gentamycin sulphate and Amoxicillin) by γ-rays doses such as (0.5, 1, 1.5, 2, 2.5 and 3 kGy) were studded. AgNPs were characterized by (UV-Vis.), (DLS),

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(FT-IR) and (TEM) analysis. Combined and individual antibacterial activities of Amoxicillin and AgNPs were investigated against different pathogenic bacterial species by measuring the (ZOI) and by determining the (MIC).
Results: This method shows that Aqueous Ag^+ ions were reduced to AgNPs when added to the cell-free supernatant of *B. megaterium* this is indicated by the color change from whitish yellow to brown and the control showed no color change. In physical method Amoxicillin was incorporated with AgNPs perfectly at 2.5kGy. The decreasing order of the average antibacterial activity against bacterial group was observed to be AgNPs > Amoxicillin > Amoxicillin + AgNPs.
Conclusion: The radiation-induced AgNPs synthesis is a simple, clean which involves radiolysis of aqueous solution that provides an efficient method to reduce metal ions. *B. megaterium* was found to be an effective biological tool for the extracellular biosynthesis of AgNPs. The bactericidal activity have proved that AgNPs in combination with amoxicillin kill bacteria at such low concentrations (units of ppm), which do not reveal acute toxic effects on human cell, in addition to overcoming resistance, and lowering cost when compared to conventional antibiotics.

Keywords: Silver nanoparticles; antibiotics; gamma irradiation; microbiological assay and biological synthesis.

ABBREVIATIONS

AgNPs= Silver Nanoparticles; SPR =Surface Plasma Resonance; NCRRT= National Center for Radiation Research and Technology; EDTA= ethylene diamine tetracetic acid; β-NADH= β-nicotinamide adenine dinucleotide reduced form; NED= N-(1-naphthyl) ethylene diamine dihydrochloride.

1. INTRODUCTION

Human beings are often infected by microorganisms such as bacteria, molds, yeasts, and viruses present in their living environments. Because of the emergence and increase in the number of multiple antibiotic-resistant microorganisms and the continuing emphasis on health-care costs, many scientists have researched methods to develop new effective antimicrobial agents that overcome the resistances of these microorganisms and are also cost-effective. Such problems and needs have led to resurgence in the use of silver-based antiseptics that may be linked to a broad-spectrum activity and considerably lower propensity to induce microbial resistance compared with those of antibiotics. [1]. It has been known that silver has strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus since ancient times. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells. It has been shown that AgNPs prepared with a variety of synthetic methods have effective antimicrobial activity. Hence, AgNPs have been applied to a wide range of healthcare products, such as burn dressings, scaffold, water purification systems, and medical devices [2].

Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities. It can be classified into intracellular and extracellular synthesis according to the location where nanoparticles are formed. The intracellular method consists

of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. The extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes. The biosynthesized nanoparticles have been used in a variety of applications including drug carriers for targeted delivery, cancer treatment, gene therapy and DNA analysis, antibacterial agents, biosensors, enhancing reaction rates, separation science, and Magnetic Resonance Imaging (MRI) [3]. In all inorganic antimicrobial agents, silver element and compounds of nanoparticles were the most extensively tested ones. Recently, silver nanoparticles were quite good antimicrobial agents against *Escherichia coli*. In addition, AgNPs has excellent properties of conformational entropy in polyvalent binding, which make it easy to attach to flexible function groups of antibiotics. Moreover, AgNPs possesses well developed surface chemistry, chemical stability, and appropriate size (20nm in diameter, 250 times smaller than a bacterium). It is able to maintain constant shape and size in solution. Thus it is a good choice to use AgNPs as inorganic nanomaterials in combination with β-lactam, as the inorganic nanomaterial against *E. coli* cells. [4]. Gamma-irradiation synthesis of metallic nanoparticles has been employed as one of the most promising method to produce AgNPs due to some important advantages. As compared to conventional chemical /photochemical techniques, the radiochemical process can be performed to reduce Ag^{+} ions at the ambient temperature without producing unwanted by-products of the reductant or using excessive reducing agents. Moreover reducing agent can be uniformly distributed in the solution and AgNPs are produced in highly pure and stable form. [5]

So, the aim of this study was to use the culture cell-free supernatant of the non-pathogenic *Bacillus megaterium* for the synthesis of AgNPs from the stand point of ease of mass production and safety in handling the organism. Also, the current work involving the optimization of medium components for nitrate reductase production by *Bacillus megaterium* to enhance the synthesis of AgNPs. Also, uses of gamma irradiation power for synthesis and incorporation of AgNPs with selected antibiotics at distinct dose. To evaluate the antimicrobial activity of the AgNPs synthesized when incorporated with Amoxicillin against selected strains of pathogenic multidrug-resistant bacteria causing life – threatening infection.

2. MATERIALS AND METHODS

2.1 Chemicals

All the media components (yeast extract, peptone and agar) from Oxoid. All chemicals and reagents used in the following experiments were of analytical grade and used without further purification.

2.2 Chemotherapeutic Substances

Chemotherapeutic agents (raw materials) used in this study such as: Amoxicillin was kindly obtained from Egyptian International Pharmaceutical Industries Company.

2.3 Irradiation Source

The process of irradiation was carried out at NCRRT. Irradiation were performed by using Cobalt 60 source (Gamma cell 4000-A-India) at a dose rate of 0.919Gy/s. and a specific activity of 3496.8 curie at the time of the experiments.

2.4 Organism and Culture Maintenance

The culture of *Bacillus megaterium* was kindly obtained from the Drug Radiation Research Department at (NCRRT), Cairo, Egypt. The culture was maintained at 35ºC in nutrient agar plate (seed media). The culture was maintained by continuous sub-culturing using seed medium every two week. *Bacillus megaterium* was grown in 50 ml medium (fermentation media for nitrate reductase production) containing different concentrations of: yeast extract, peptone and KNO_3, in 250ml Erlenmeyer flask at 30ºC with shaking (200 rpm) for 24hrs. Using (LAB-Line Orbit Environ) shaker.as shown in Table 5.Cell free supernatant was separated by centrifugation using (Hettich Universal 16R cooling centrifuge) at 6,000 rpm for 10minutes at 6ºC.

2.5 Estimation of Nitrate Reductase Enzyme Activity

Nitrate reductase activity was estimated by modified procedure based on the method of [6]**.** In our study, the five factors for optimization of fermentation processes (Yeast extract, Peptone, KNO_3, Temperature and Incubation period) were optimized using (RSM) to improve the microbial production of Nitrate reductase enzyme. Nitrate erductase enzyme activity was measured according to the method of reductase assay by UV-Vis spectroscopy. The cell free supernatant obtained was used as the crude enzyme. The protocol use 25mM potassium phosphate buffer with 30mM potassium nitrate and 0.05mM ethylene diamine tetracetic acid (EDTA) at pH 7.3 as the enzyme substrate. Enzyme (100 µl) was allowed to react with 1.8ml of substrate solution and the reaction was supported by 100 µl of 2.5mM β-nicotinamide adenine dinucleotide reduced form (β-NADH). The reaction mixture was incubated at 30ºC for 5min. Then the reaction was terminated immediately by adding and swirling 1ml of 58mM sulphanilamide solution in 3M HCl. Then 1ml of 0.77mM N-(1-naphthyl) ethylenediamine dihydrochloride solution (NED) was added and mixed by swirling. The color formation was noticed after 5 min incubation of the mixture. The absorbance was recorded at 540nm. Unit is defined as micromoles of nitrite produced per min [6].

2.6 Synthesis of Silver Nanoparticles

Synthesis of AgNPs was carried out according to method described by [5-7].with slight modification.

2.6.1 Biological synthesis of agnps

In Erlenmeyer flask, cell supernatant of optimum medium mixed with 1mM silver nitrate ($AgNO_3$) and incubates for 5min. The absorption spectrum of the sample was recorded on JASCO V-560 UV-visible spectrometer operating at a resolution of 1nm.

2.6.2 Synthesis of AgNPs by gamma irradiation

Colloidal silver nanoparticles were prepared as follows:
The solution was prepared by the addition of 1mM $AgNO_3$ and 2.7mM of Antibiotics then the solution was irradiated at different doses (0.5, 1, 1.5, 2, 2.5 and 3kGy) at a dose rate of 0.919Gy/s. and a specific activity of 3496.8 curie with a control solution.

2.7 Characterization of Silver Nanoparticles

The produced AgNPs were characterized by the following:

2.7.1 UV-visible spectrophotometer (UV-Vis)

UV-Vis Spectra of AgNPs was recorded as a function of wavelength using JASCO V-560. UV-Vis is spectrophotometer from 200-900nm at a resolution of 1nm.

2.7.2 Dynamic Light Scattering (DLS)

Average particle size and size distribution were determined by PSS-NICOMP 380-ZLS particle sizing system St. Barbara, California, USA. Before measurements the samples were diluted 10 times with deionized water. 250µl of suspension were transferred to a disposable low volume cuvette. After equilibration to a temperature 25ºC for 2min, five measurements were performed using 12 runs of 10s each.

2.7.3 Fourier Transform Infrared spectrometer (FT-IR)

FT-IR measurements were carried out in order to obtain information about chemical groups present around AgNPs for their stabilization and conclude the transformation of functional group due to reduction process. The measurements were carried out using JASCO FT-IR-3600 infra-red spectrometer by employing KBr Pellet technique.

2.7.4 Transmission Electron Microscopy (TEM)

The size and morphology of the synthesized nanoparticles were recorded by using TEM model JEOL electron microscopy JEM-100 CX. TEM studies were prepared by drop coating silver nanoparticles onto carbon-coated TEM grids. The Film on the TEM grids were allowed to dry, the extra solution was removed using a blotting paper.

2.8 Incorporation of AgNPs with Selected Antibiotics

Silver nanoparticles were combined with antibiotics as following:

AgNPs which synthesized by *Bacillus megaterium* supernatant are mixing with amoxicillin by added 2.7mM of antibiotic with 10ml of stock solutions of (105ppm) AgNPs and incubates at room temperature for 10min. Also, 1mM $AgNO_3$ solution which irradiated (at different gamma doses) with 2.7mM Amoxicillin powder was incorporate with it by the irradiation power.

2.9 Antimicrobial Study of the Synthesized Silver Nanoparticles, Selected Antibiotics and the Combined AgNPs with Antibiotics

2.9.1 Determination of zone of inhibition (ZOI)

The synthesized AgNPs, Amoxicillin and a mixed solution of both respectively were tested for their antimicrobial activity by the agar well diffusion method [8] against different kinds of pathogenic multidrug resistance bacteria. Wells of 10mm diameter were bored into the nutrient agar medium using crock poorer. Using micropipette, 100µl AgNPs (105, 52.5, 26.25, 13.125 and 6.5ppm), Amoxicillin solution (2.7mM) and a mixed solution of Amoxicillin

in a different ppm of AgNPs were added into each well. After incubation at 37ºC for 24hr. the different levels of zone of inhibition were measured and interpreted using the CLSI zone diameter interpretive standards [5,9]. Tetracycline (Antibacterial agent) served as positive control for antimicrobial activity, while the filtrate alone (without AgNPs) was used as negative control. The determinations were done in triplicates and the mean values ± SD (standard deviation) were presented.

2.9.2 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) determination were performed in Luria Bertani (LB) broth in duplicate using serial two-fold dilutions of (AgNPs, Amoxicillin and mixed solution of both, along with positive control well (the microorganism and the nutrient) and negative control one (the nutrient only) [5,9]. The MIC was determined after 24hrs of incubation at 37ºC with initial inoculums of 0.1 OD at 600nm. MIC can by reading by two methods:

a) - Visually by comparison with the drug free controls.
b) - With ELISA plate reader at a wavelength of 620nm.

2.10 Statistical Analysis

The results of optimization of medium components for nitrate reductase production were analyzed by using the software version 6.0 (Minitab.11 MP), Also All tests were carried out in duplicate. For comparison and statistical analysis, each of these tests was considered as an individual observation; therefore, two observations were obtained for each combination of isolate and antibiotic agent.

3. RESULTS AND DISCUSSION

3.1 Synthesis of Silver Nanoparticles (AgNPs)

3.1.1 Biosynthesis of silver nanoparticles

Aqueous Ag^+ ions were reduced to AgNPs when added to the cell free supernatant of *Bacillus megaterium* this is indicated by the color change from whitish yellow to brown and the control showed no color change. The appearance of brown color in $AgNO_3$ treated flask suggested the formation of AgNPs and the color change is attributed to the surface plasma resonance (SPR). This change in color of the cell free supernatant was previously linked with the formation of AgNPs and depicts the excitation of Surface Plasmon Vibrations in the nanoparticles .Silver nanoparticles are extraordinarily efficient at absorbing and scattering light and, unlike many dyes and pigments, have a color that depends upon the size and the shape of the particles. The strong interaction of the AgNPs with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when excited by light at specific wavelengths known as a surface plasma resonance (SPR) [10].

The possible mechanism of AgNPs formation by *Bacillus megaterium* reveals to Nitrate Reductase Enzyme [5]. This enzyme is induced by nitrate ions and reduces silver ions to metallic silver. And the mechanism that may involve the reduction of silver ions is the electron shuttle enzymatic metal reduction process. NADH and NADH-dependent nitrate reductase enzymes are important factors in the biosynthesis of metal nanoparticles.

B. megaterium known to secrete the cofactor NADH and NADH-dependent enzymes especially nitrate reductase, which might be responsible for the bio reduction of Ag^+ to Ag^o and the subsequent formation of silver nanoparticles. So, characterization was done by spectrophotometric analysis revealed a peak at wavelength 415nm indicating the presence of spherical or roughly AgNPs [11]. So, the scope of the present study was based upon exploring the capabilities of bacterial strains *B.megaterium* to synthesize AgNPs along with their biomedical application in controlling infectious bacteria. In addition, these nanoparticles alone and also incorporation with Amoxicillin proved to be effective in controlling resistant bacteria such as Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* & Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa.*

3.1.2 Synthesis of silver nanoparticles by gamma irradiation

The mechanism of radio lytic reduction of aqueous solution is carried out by organic radicals formed. Antibiotic molecules play an important role in scavenging the free radicals and converted into organic radicals [11]. In this study, the aqueous solution of metal salt is exposed to γ-rays; the species hydrated electron and hydrogen atoms arising from radiolysis of water are strong reducing reagents as show in Equation 1. And they reduce the metal ion to zero valent state.

$$* H_2O \xrightarrow{\gamma\text{-irradiation}} e^-_{aq,}\, H_3O^+,\, H^{\cdot},\, OH^{\cdot},\, H_2^{\cdot},\, H_2O_2^{\cdot} \qquad (1)$$

The radiolytic method is particularly suitable for generation of metal NPs in solution, because the radiolytically generated species exhibit strong reducing power and reduction of metal ions occur at each encounter.$AgNO_3$ separated to Ag^+ and NO_3^- ions in the aqueous solution as show in Equation 2.

$$*AgNO_3 \xrightarrow{\text{aqueous solution}} Ag^+ + NO_3^- \qquad (2)$$

The solvated electrons, i.e, e^-_{aq} and H atoms are strong reducing agents; therefore, in the following step, they easily reduced silver ions down to the zero valent state as show in Equation 3 and 4.

$$*Ag^+ + e^-_{aq,} \xrightarrow{\text{Reduction}} Ag^0 \qquad (3)$$

$$*Ag^+ + H^{\cdot} \xrightarrow{\text{Reduction}} Ag^0 + H^+ \qquad (4)$$

Silver atoms formed by the irradiation tended to coalesce into oligomers (Equation 5), which progressively grew into large clusters (Equation 6). The aqueous electrons reacted with the Ag^+ clusters to form relatively stabilized Ag clusters (Equation 7). [11,12]**.**

$$*Ag^0 + Ag \longrightarrow Ag^+_2 \qquad (5)$$

$$*nAg^+ + Ag^+_2 \longrightarrow (Ag)_n^+ \qquad (6)$$

$$*(Ag)_n^+ + ne^-_{aq,} \longrightarrow (Ag)_n \qquad (7)$$

Table 1 explains the selected does for AgNPs formation and incorporation with amoxicillin. From the obtained results it was noted that AgNPs were synthesized by gamma irradiation in the present of Amoxicillin as a reducing and stabilizing agent.

Table 1. The result of irradiated $AgNO_3$ solution with different antibiotics at different doses for AgNPs formation and incorporation with antibiotics

Doses / Antibiotics	0.5 kGy	1.0 kGy	1.5 kGy	2.0 kGy	2.5 kGy	3.0 kGy
Amoxicillin	Positive	Positive	Positive	Positive	Positive	Positive
Gentamycin	Negative	Negative	Negative	Negative	Negative	Negative
Cefotaxime	Negative	Negative	Negative	Negative	Negative	Negative

3.2 Characterization of Silver Nanoparticles (AgNPs)

3.2.1 UV-visible spectrophotometer (UV-Vis)

The dispersion of silver nanoparticles displays intense colors due to the Plasmon resonance absorption. The surface of a metal is like plasma, having free electrons in the conduction band and positively charged nuclei. Surface Plasmon Resonance is a collective excitation of the electrons in the conduction band; near the surface of the nanoparticles. Therefore, metallic nanoparticles have characteristic optical absorption spectrum in the UV-visible region [13]. As shown in Fig. 1, UV-Visible spectrum of AgNPs Synthesized by gamma irradiation at 2.5kGy. Also, Table 2 exhibits the maximum absorption of prepared sliver nanoparticles in different gamma irradiation doses.

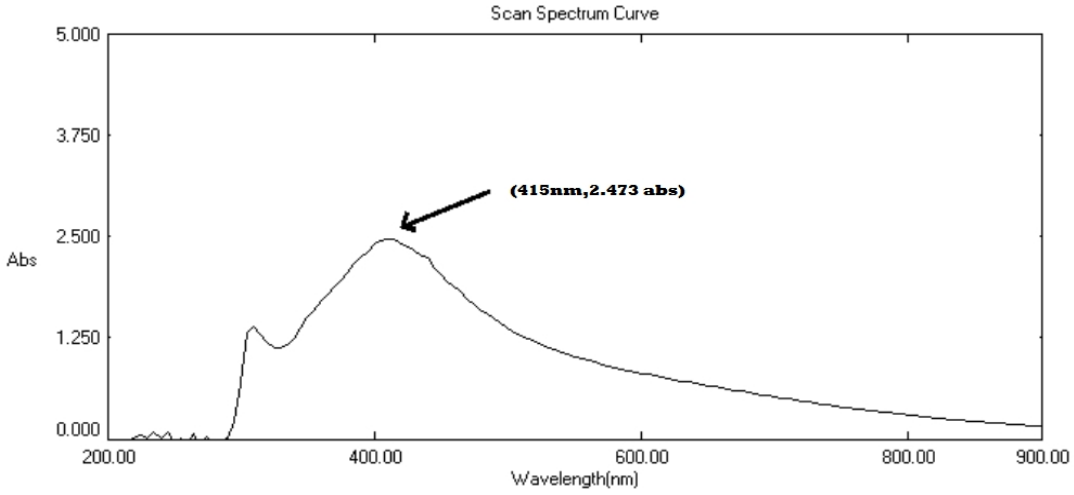

Fig. 1. UV-visible spectrum of AgNPs Synthesized by gamma irradiation in the presence of amoxicillin at a dose of 2.5kGy

Table 2 showed that the O.D of AgNPs formation is increased with the increase in gamma irradiation and above 2.5kGy there is a significant decrease in the maximum absorption.

HPLC analysis is used to explain that Amoxicillin still present in the solution mixtures which then explain the dual effect of the antibiotic present with the synthesized AgNPs against

selected pathogenic microorganisms which increase the synergistic effect of both. The following Figs. 2a and 2b explain that amoxicillin does not break down as the follow

Table 2. Show the UV-Visible Spectroscopy result of AgNPS Synthesized by Gamma Irradiation

Gamma Irradiation Doses (kGy)	Wavelengths (nm)	Maximum absorption (O.D.)
0.5	410.00	1.971
1	400.00	2.038
1.5	390.00	2.140
2	390.00	2.265
2.5	415.00	2.473
3	415.00	2.356

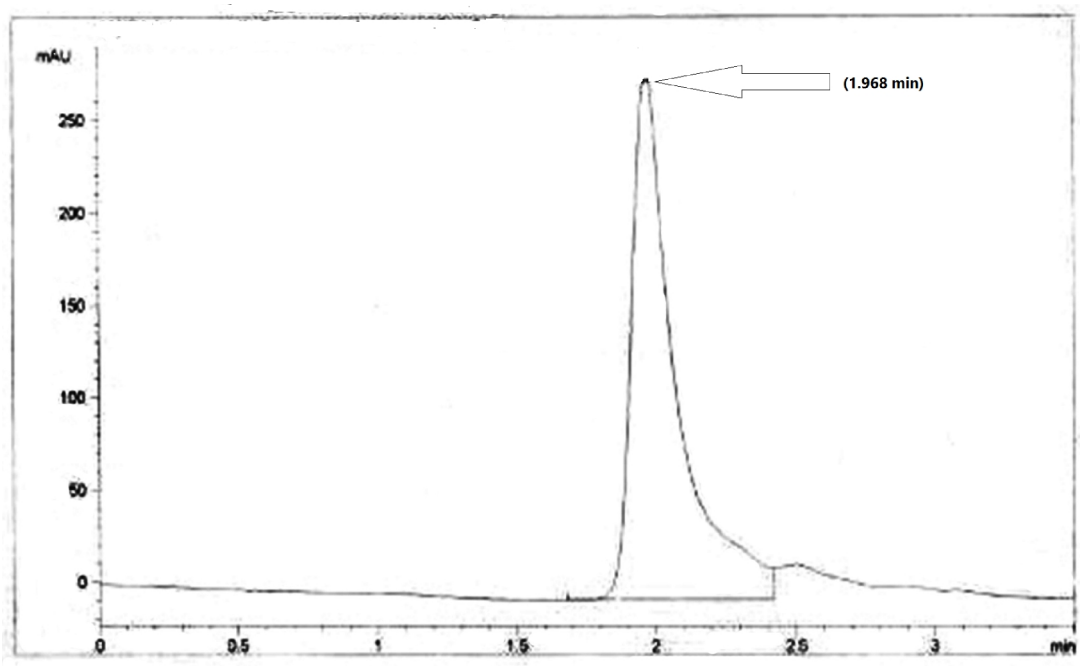

Fig. 2a. HPLC analysis of non-Irradiated Amoxicillin (as a control sample)

In the Figs. 2a and 2b: Amoxicillin which non irradiated was separated by HPLC at retention time (1.968min) and irradiated Amoxicillin was separated by HPLC at retention time (2.062) which means there is no differences in the position of two peaks in the scales and this indicated that irradiation power does not break down Amoxicillin which irradiated with $AgNO_3$ (1mM) for the synthesis of incorporated AgNPs with Amoxicillin and the antimicrobial activity of irradiated samples ($AgNO_3$+Amoxicillin) returned to the dual effect of both antibiotic and synthesized AgNPs. Also, Fig. 3. Show UV-Visible spectrum of AgNPs Synthesized by *B. megaterium.*

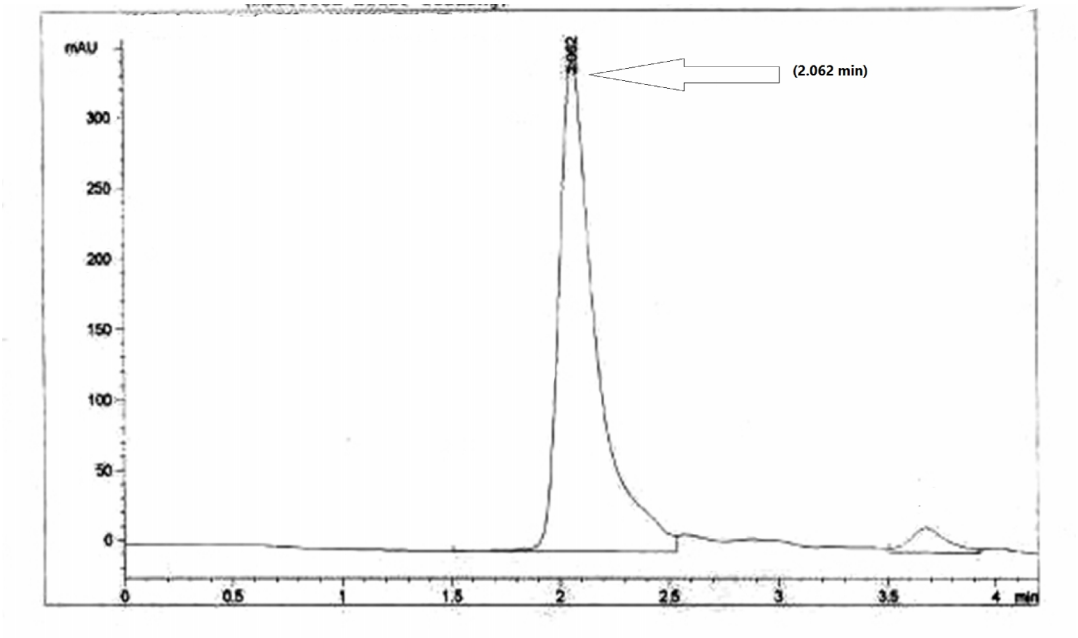

Fig. 2b. HPLC analysis of irradiated Amoxicillin with 1mM $AgNO_3$ at 2.5kGy

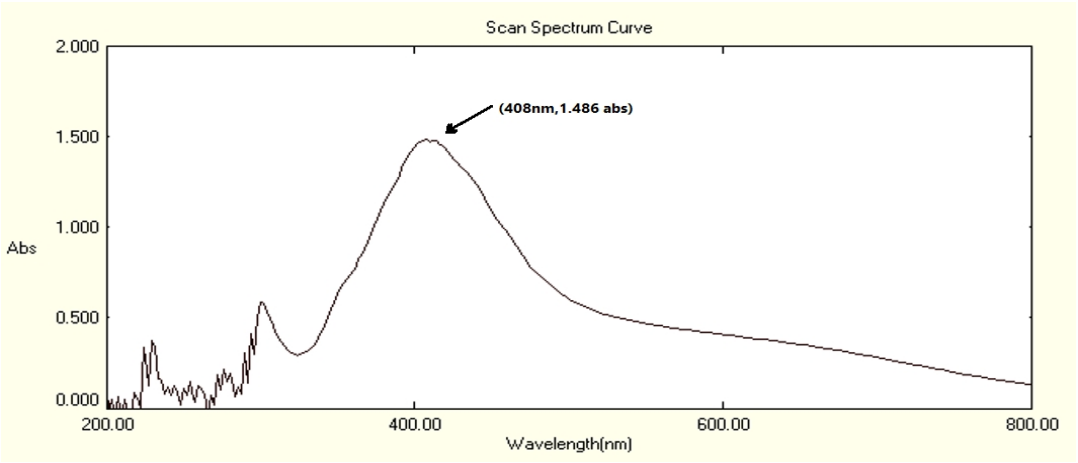

Fig. 3. UV-Visible spectrum of AgNPs Synthesized by cell-free supernatant of *B. megaterium*

The presence of the signature peaks of amino acid supports the presence of proteins in cell-free filtrate as observed in UV-Visible spectra. The absorption spectrum of the silver nanoparticles, shown in all the figures, indicates the production of the nanoparticles where the presence of a Plasmon absorption band at 415 nm is characteristic of silver nanoparticles [11].

3.2.2 Dynamic Light Scattering (DLS)

The average particle size was determined by DLS method and was found to be 10.9nm as shown in Fig. 5 in AgNPs which synthesized by the radio lytic reaction of gamma radiation at 2.5kGy. Also Fig. 4 show the particle size of AgNPs which synthesized by *B. megaterium* which found to be 19nm.

3.2.3 Transmission Electron Microscopy (TEM)

TEM examination of the solution containing AgNPs which synthesized by cell-free supernatant of *B. megaterium* demonstrated spherical particles within nano range from 9.3 nm to 20.3nm with the main diameter of 14.24nm as shown in Fig. 6. The particle size obtained from DLS measurement with main diameter of 19 nm Fig. 4 is obviously larger than TEM result (14.24nm) as shown in Fig. 6, because DL analyze measures the hydrodynamic radius which take into consideration the native protein on the surface of silver nanoparticles as well.

Also, TEM examination of the solution containing AgNPs which synthesized by gamma irradiation demonstrated spherical particles within nano range from 8.2nm to 22.7nm with the main diameter of 13.71nm as shown in Fig. 7.

3.2.4 Fourier Transform Infrared spectrometer (FT-IR)

It was observed From the FT-IR spectrum of AgNPs which synthesized by cell-free supernatant of *B. megaterium* that the bands at 1631.48cm-1 corresponding to a primary amine (NH band), 1400.07cm^{-1} corresponding to a secondary amine (NH_2 band) and 1157.08 cm^{-1} corresponding to a primary amine (NH stretch vibrations of the proteins) as shown in Fig. 8a, 8b and Table 3. The positions of these bands were close to that reported for native proteins. The FT-IR results indicate that the secondary structure of proteins was not affected as a consequence of reaction with Ag^+ ions or binding with AgNPs [5,12]. This evidence suggests that the release of extracellular protein such as nitrate reductase enzymes could possibly perform the formation and stabilization of AgNPs in aqueous medium [11].

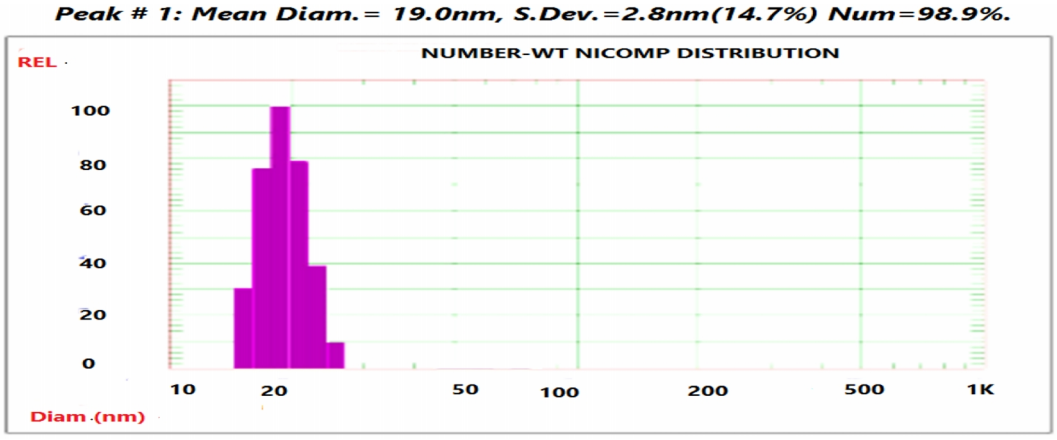

Fig. 4. Particle Size Distribution by DLS of AgNPs synthesized by cell-free supernatant of *B. megaterium*

Also, it was observed from FTIR measurements of irradiated Amoxicillin at 2.5kGy (control sample) and AgNPs which synthesized by gamma radiation at 2.5kGy and incorporated with amoxicillin that there is no differences in the position of peaks and slightly changes in %T which indicated that AgNPs incorporated or capped physically by the function group of amoxicillin [11]. As shown in Figs. 9a and 9b and Table 4.

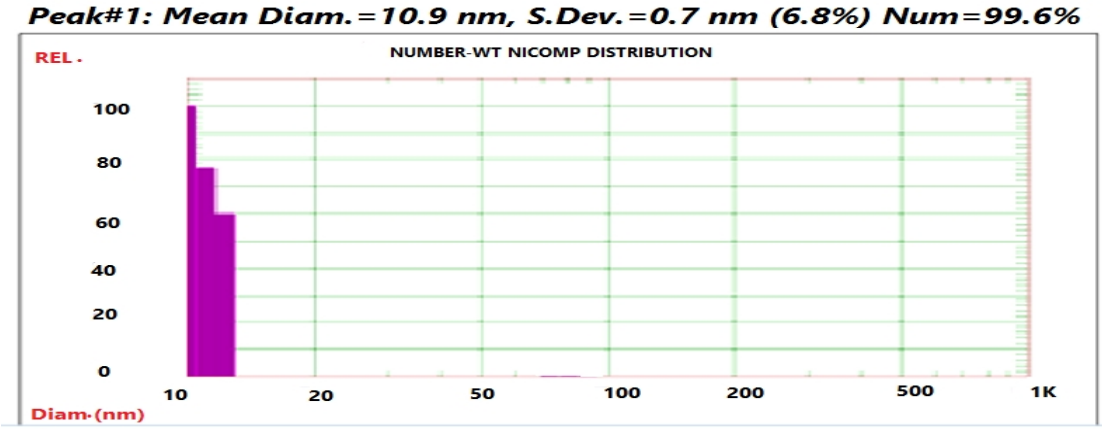

Fig. 5. Particle Size Distribution by DLS of AgNPs synthesized by gamma radiation in the presence of amoxicillin at dose of 2.5kGy

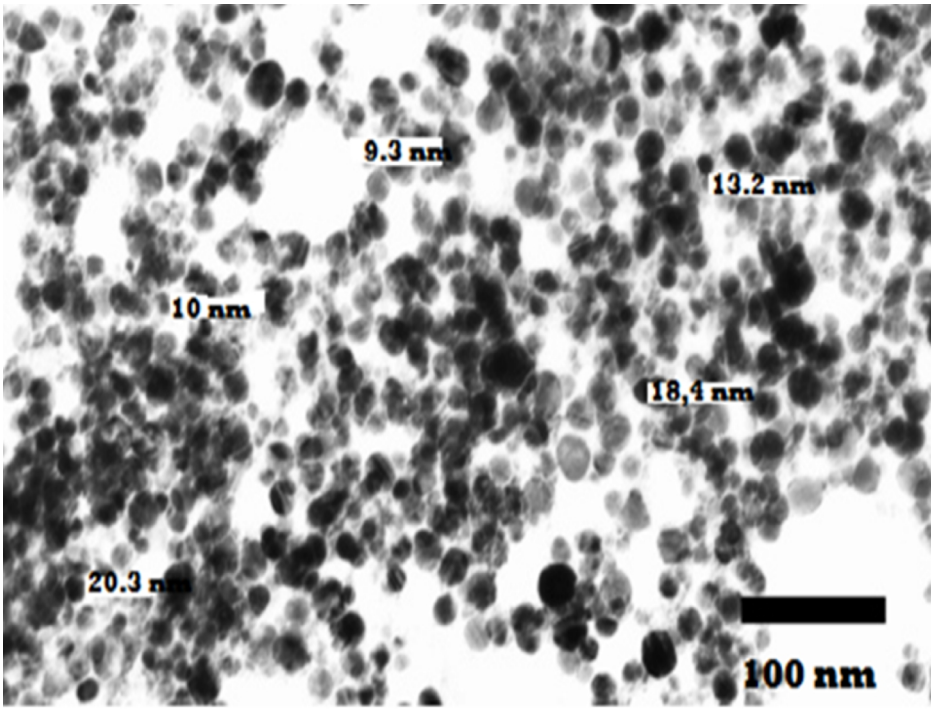

Fig. 6. TEM image for AgNPs synthesized by cell-free supernatant of *B. megaterium*

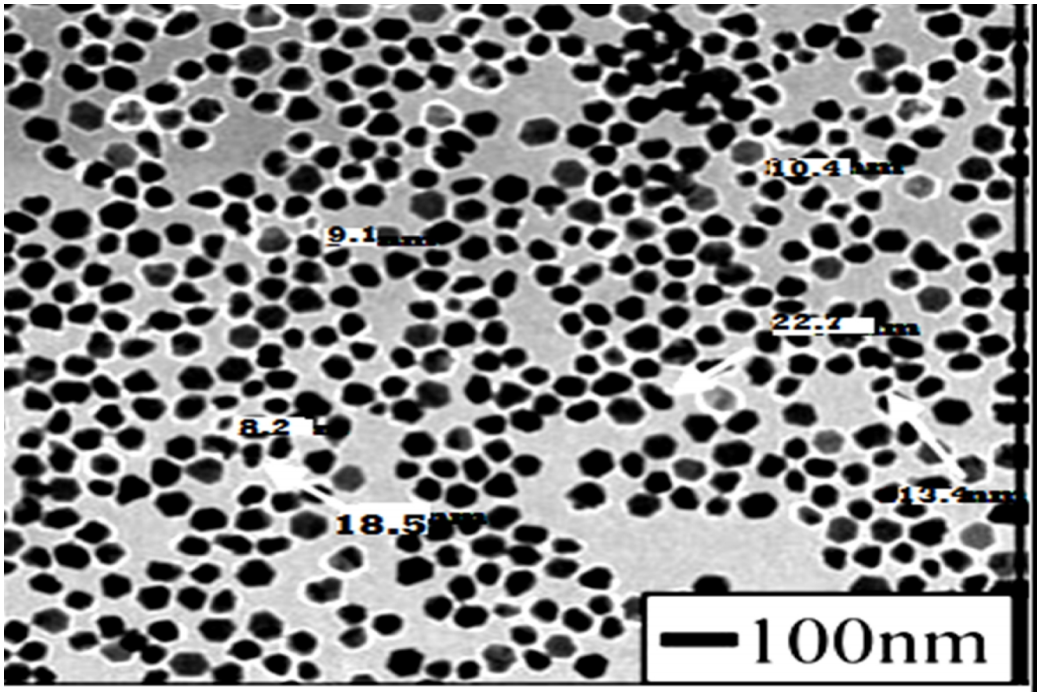

Fig. 7. TEM image for silver nanoparticles synthesized by gamma irradiation in the presence of amoxicillin at dose of 2.5kGy

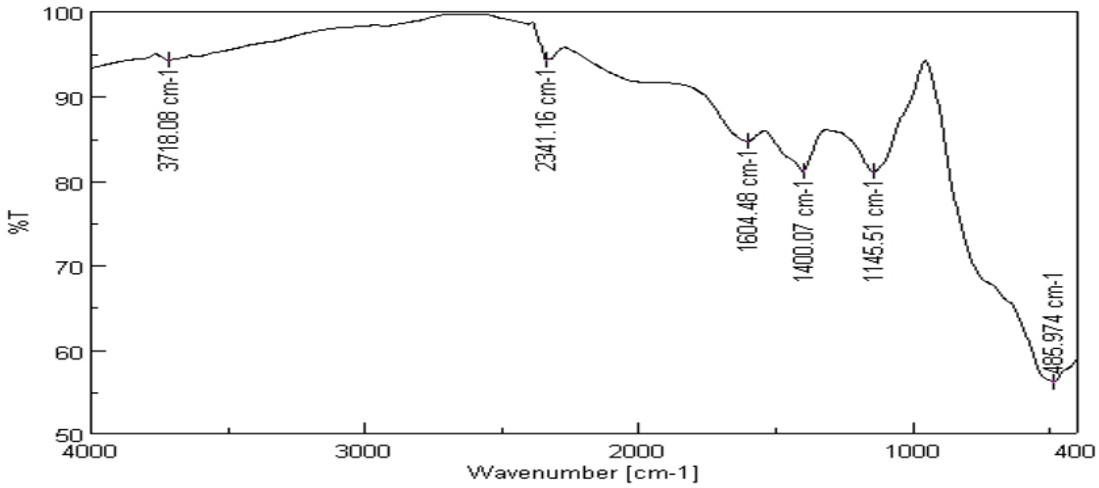

Fig. 8a. FT-IR spectrum of fermented extract of *Bacillus megaterium*

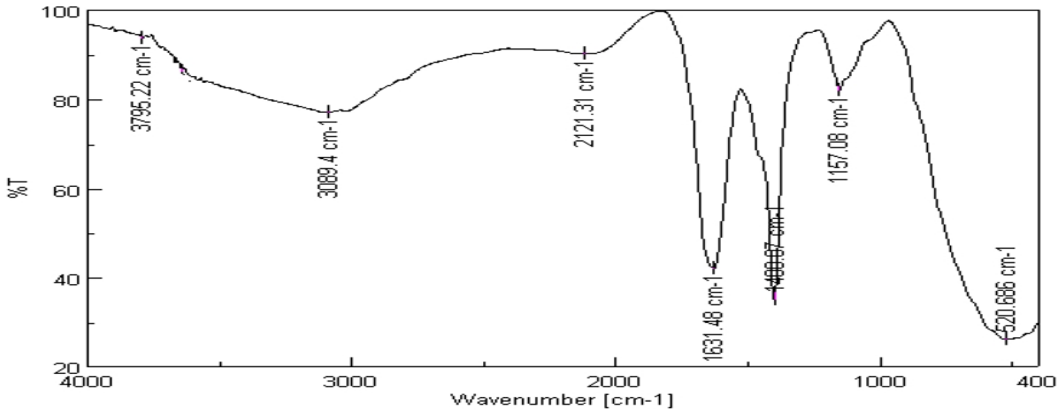

Fig. 8b. FT-IR spectrum of fermented extract with silver nanoparticles

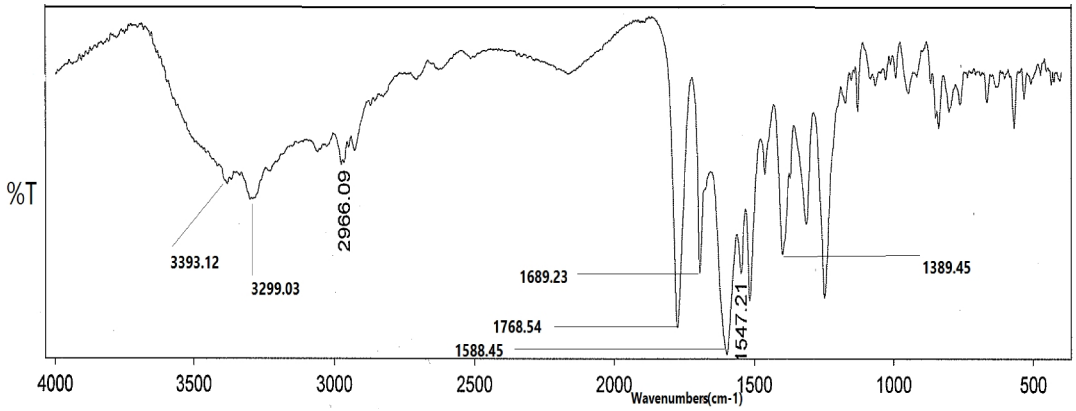

Fig. 9a. FTIR analysis of irradiated amoxicillin at 2.5 kGy (control sample)

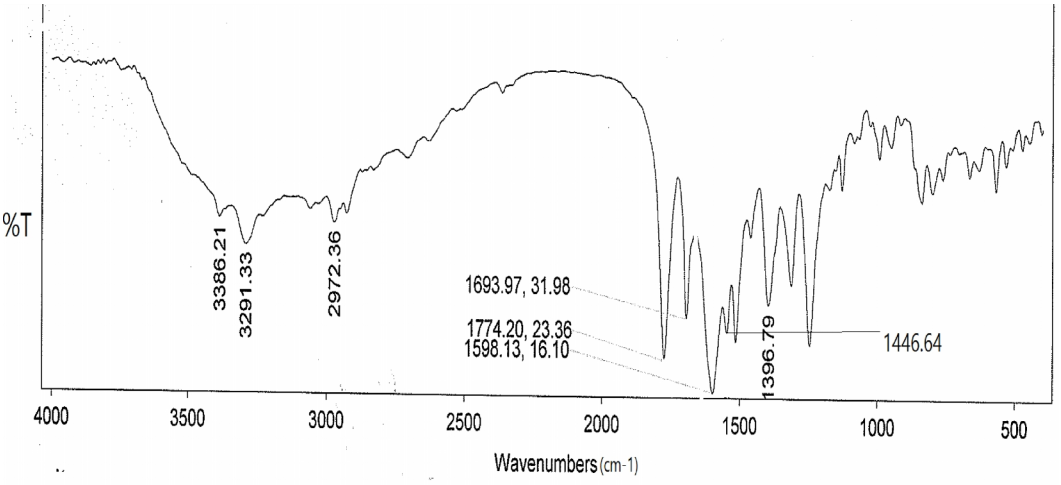

Fig. 9b. FTIR analysis of AgNPs synthesized and incorporated with amoxicillin by gamma irradiation at 2.5kG

Table 3. Wavenumber of characteristics bonds and corresponding assignments for *B. megaterium* without and with AgNPs

Peak number	Extract ƛ (cm^{-1})	Extract + AgNPs ƛ (cm^{-1})	Comment
1	3718.08	3795.22	Corresponding to OH band vibration [14].
2	-------	3089.4	The broad peaks are characteristic to the presence of $–NH_2$ amino group and –OH stretching groups in alcoholic and phenolic compounds. The presence of this peak may be due to binding of Ag ions to OH group. [14,15].
3	2341.16	2121.31	Corresponding to aliphatic C-H stretching [14].
4	1604.48	1631.48	Characteristic to the carbonyl group. This red shift may indicate oxidation of carbonyl group during the reaction and hence reducing silver to silver nanoparticles. [15].
5	1400.07	1400.07	May be ascribed for the presence of Secondary amine group C-NH stretching. [16].
6	1145.51	1157.08	Corresponding to a primary amine (NH stretch vibrations of the proteins) [14].

Table 4. Wavenumber of characteristics bonds and corresponding assignments for Amoxicilin without and with AgNPs

Peak number	Amoxicillin ƛ (cm^{-1})	Amoxicillin + AgNPs ƛ (cm^{-1})	Comment
1	3393.12	3386.21	Corresponding to OH band in Amoxicillin function group [14]. And also, corresponding to (O-H, N-H stretching vibration) [20].
2	3299.03	3291.33	The broad peaks are characteristic to the presence of $–NH_2$ amino group and –OH stretching groups in amoxicillin. [14,15 and 20].
3	2966.09	2972.36	Corresponding to CH_3 & CH_2 function groups of Amoxicillin [19].
4	1768.54	1774.20	Corresponding to C=O-H function groups of amoxicillin [19]. And Also, Corresponding to (C=O stretching of β-lactamic) [20].
5	1689.23	1693.97	Characteristic to the carbonyl group. This red shift may indicate oxidation of carbonyl group during the reaction and hence reducing silver to silver nanoparticles [15]. And Also, Corresponding to (C = O stretching of amide) [20].
6	1588.45	1598.13	Corresponding to (asymmetric stretching of carboxylate) [20].
7	1547.21	1446.64	Corresponding to NH & NH_2 function groups of Amoxicillin. [19]
8	1389.45	1396.79	Corresponding to stretching vibration of C-O bending [20].

3.3 Optimization of Medium Components for Nitrate Reductase Production

In the present study, the highest value of nitrate reductase activity obtained was 680.89 U/ml which was observed in the 13th run with the following condition;(media containing: (%) yeast extract:0.15, pepton:0.25, KNO_3:0.1,temp,:30ºC and incubation period one day). The experiment conditions and the results of nitrate reductase activity are shown in Table 5.

Table 5. Combination of variables and Response in RSM design

No. of runs	Yeast extract (%)	Peptone (%)	Pot. nitrate (%)	Tem, (ºC)	Incubation period (Day)	OD at ƛ 540nm of AgNPs	Enzyme activity (U/ml)
1.	0.3	0.3	0.1	30	1	3.173	556.35
2.	0.3	0.3	0.1	35	1	1.990	420.86
3.	0.3	0.3	0.1	30	2	1.510	411.22
4	0.3	0.3	0.1	35	2	0.254	146.30
5	0.3	0.3	0.2	30	1	2.040	612.73
6	0.3	0.3	0.2	35	1	3.281	630.86
7	0.3	0.3	0.2	30	2	3.243	600.06
8	0.3	0.3	0.2	35	2	0.703	200.73
9	0.3	0.5	0.1	30	1	3.020	580.69
10	0.3	0.5	0.1	35	1	3.000	532.32
11	0.3	0.5	0.1	30	2	0.425	180.23
12	0.3	0.5	0.1	35	2	0.101	65.23
13	0.3	0.5	0.2	30	1	3.300	680.89
14	0.3	0.5	0.2	35	1	0.201	100.32
15	0.3	0.5	0.2	30	2	0.222	136.38
16	0.3	0.5	0.2	35	2	0.348	166.68
17	0.5	0.3	0.1	30	1	0.346	165.64
18	0.5	0.3	0.1	35	1	1.241	396.02
19	0.5	0.3	0.1	30	2	3.216	663.60
20	0.5	0.3	0.1	35	2	2.680	425.77
21	0.5	0.3	0.2	30	1	1.250	400.03
22	0.5	0.3	0.2	35	1	0.291	155.36
23	0.5	0.3	0.2	30	2	1.640	406.89
24	0.5	0.3	0.2	35	2	1.990	420.86
25	0.5	0.5	0.1	30	1	0.077	35.23
26	0.5	0.5	0.1	35	1	0.753	206.35
27	0.5	0.5	0.1	30	2	1.255	400.03
28	0.5	0.5	0.1	35	2	0.210	118.30
29	0.5	0.5	0.2	30	1	0.612	196.86
30	0.5	0.5	0.2	35	1	0.709	201.99
31	0.5	0.5	0.2	30	2	1.022	308.96
32	0.5	0.5	0.2	35	2	0.218	122.36

- 0.5% means 0.25mg of yeast extract and peptone in 50ml Deionized water.
- 0.3% means 0.15mg of yeast extract and peptone in 50ml Deionized water.
- 0.2% means 0.1mg of potassium nitrate in 50ml Deionized water.
- 0.1% means 0.05mg of potassium nitrate in 50ml Deionized water.

The result in Table 5 demonstrated that the nitrate reductase activity was significantly affected by peptone and potassium nitrate. Peptone at high level 0.5% showed higher nitrate activity. While at high concentration of KNO_3 (0.2%) showed higher nitrate activity. Temperature at low level 30ºC showed higher nitrate reductase activity. Nitrate reductase activity was higher at the low level of incubation period (1 day).The standard (Sodium nitrite) show OD at ƛ 540 nm equal to (0.534). The biggest advantage of this study based on enzyme is the development of a new approach for the synthesis of nanomaterial [5].

3.3.1 Normal probability plot

The normal probability plot show that the results are normally distributed which mean that the whole experiment is stable as shoe in Fig. 10 and Table 6 explain regression coefficient for AgNPs concentration under physical parameters.

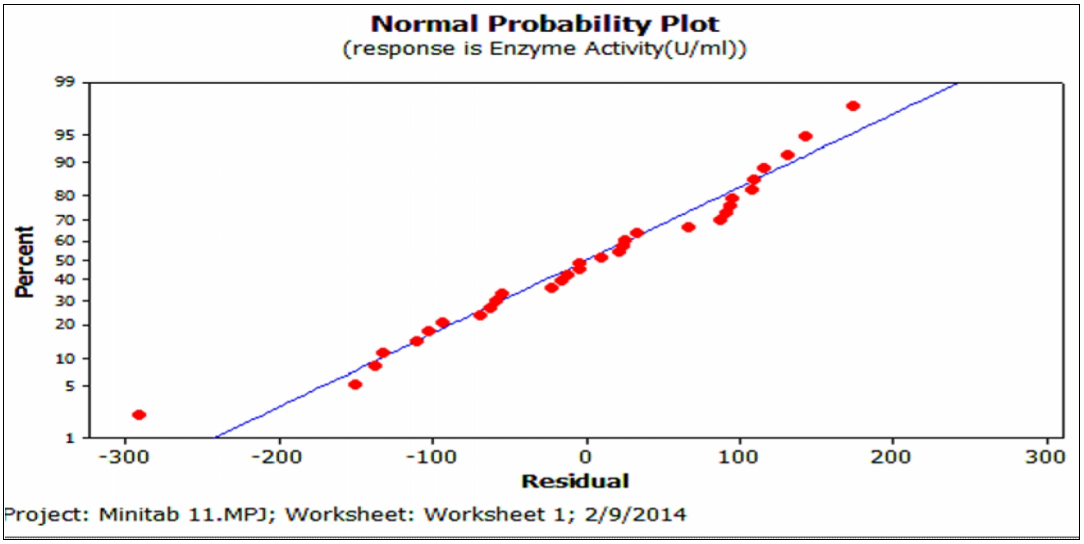

Fig. 10. Normal probability plots

3.3.2. Normal plot of the standardized effects

The results of the statistical analysis show the factors which have significant effect when $p<0.05$ and non-significant effect at $p>0.05$. It was found that the significant factors are Yeast Extract%, Temperature, Peptone%, and Incubation Period as show in Fig. 11.

3.4 Antimicrobial Activities

The efficacy of antibiotic, synthesized nanoparticles, and their combination in term of inhibition (mm) was measured against four different bacteria as shown in Table 7. The decreasing order of the average antibacterial activity of antibiotic, AgNPs and their incorporation against bacterial group was observed to be AgNPs > Amoxicillin > Amoxicillin + AgNPs. The average antibacterial activity of the amoxicillin, AgNPs and their combination against bacterial activities ranged from 2 to 38mm (zone of inhibition).It was observed that as a concentration of synthesized AgNPs increased the inhibition zone of its combination with amoxicillin increased. In the case of AgNPs, mild bactericidal activities were observed in

term of zone of inhibition ranging from 2 to 10mm. and these will be accomplished by the standard antibacterial antibiotics as show in Table 8. Using the standard dilution macro method, the MIC of the Amoxicillin, synthesized AgNPs and their combination for the selected strains are summarized in Table 9.

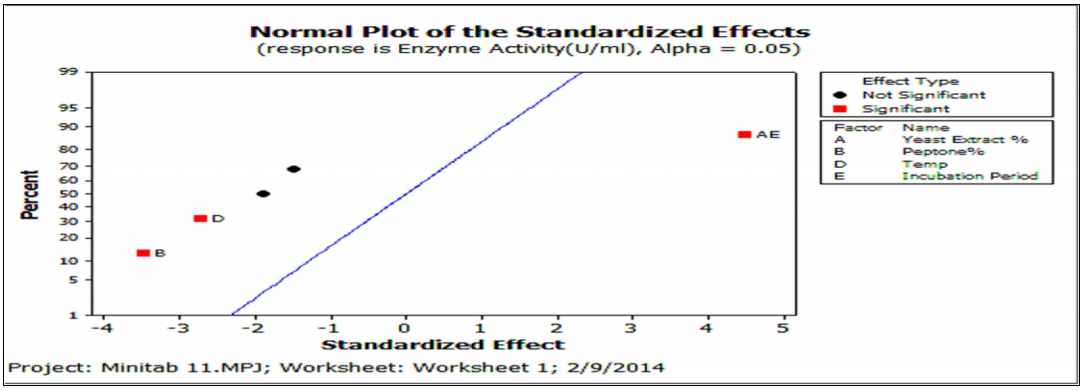

Fig. 11. The significant factors in AgNPs formation

Table 6. Regression Coefficient for AgNPs concentration under physical parameters

Term	Effect	Coef	SE Coef	T-Value	P-Value
Constant		332.69	25.66	12.97	0.000
Yeast Extract %	-87.35	-43.67	25.66	-1.70	0.108
Peptone%	-161.28	-80.64	25.66	-3.14	0.006
Pot. Nitrate %	2.36	1.18	25.66	0.05	0.964
Temp	-126.59	-63.30	25.66	-2.47	0.025
Incubation Period	-68.68	-34.34	25.66	-1.34	0.199
Yeast Extract %*Peptone%	-19.23	-9.62	25.66	-0.37	0.713
Yeast Extract %*Pot. Nitrate %	-27.07	-13.53	25.66	-0.53	0.605
Yeast Extract %*Temp	60.31	30.16	25.66	1.18	0.257
Yeast Extract %*Incubation Period	207.34	103.67	25.66	4.04	0.001
Peptone%*Pot. Nitrate %	-27.86	-13.93	25.66	-0.54	0.595
Peptone%*Temp	0.88	0.44	25.66	0.02	0.987
Peptone%*Incubation Period	-60.88	-30.44	25.66	-1.19	0.253
Pot. Nitrate %*Temp	-41.36	-20.68	25.66	-0.81	0.432
Pot. Nitrate %*Incubation Period	-8.33	-4.17	25.66	-0.16	0.873
Temp*Incubation Period	-53.55	-26.78	25.66	-1.04	0.312

S = 145.132 PRESS = 1348050 R-Sq = 72.53% R-^{Sq}(pred) = 0.00% R-^{Sq}(adj) = 46.78%

Table 7. Combined and individual efficacy of Amoxicillin and AgNPs against selected bacteria

Tested strains	Diameter of inhibition zones (mm) produced by AgNPs (105 ppm), Amoxicillin and its combination			Diameter of inhibition zones (mm) produced by AgNPs (52.5 ppm), Amoxicillin and its combination			Diameter of inhibition zones (mm) produced by AgNPs (26.25 ppm) , Amoxicillin and its combination			Diameter of inhibition zones (mm) produced by AgNPs (13.125 ppm), Amoxicillin and its combination			Diameter of inhibition zones (mm) produced by AgNPs (6.5 ppm) , Amoxicillin and its combination		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
E. coli ATCC 7839	25±1	10±2	38±1.5	25±2	8±1	32±2	25±2	5±1.5	32±3	25±2	2±1	30±2	25±1.5	2±2	30±0.5
S. aureus ATCC 6538 (MRSA)	25±2	2.5±1	32±2	25±3	2±1	30±2	25±1	2±2	27±1	25±1	2±2	26±2	25±1.5	2±2	26±1.5
P. aeruginosa ATCC 9027	15±1.5	2±2	25±1	15±2	2±2	23±2	15±2	2±2	25±2	15±1	2±1	20±2	15±1	2±2	19±1
B. subtilis ATCC 6633	18±2	7±2	26±1	18±1	7±1.5	24±2	18±2	5±1	23±1	18±2	5±1	23±2	18±1	3±1	22±1

**A: Amoxicillin alone;*B: AgNPs alone. *C: Amoxicillin+ AgNPs. *Concentration of Amoxicillin (2.7 mM); *E. coli: Escherichia coli, S. aureus: Staphylococcus aureus, P. aeruginosa: Pseudomonas aeruginosa and B. subtilis: Bacillus subtilis*

Table 8. The antimicrobial activity (inhibition zone in mm) of Tetracycline Standard antibacterial agent against different strains of pathogenic bacteria

Tested Strains	(Tetracycline) Standard antibacterial agent Diameter of inhibition zones (mm) [14]
E. coli ATCC 7839	32 mm
S. aureus ATCC 6538 (MRSA)	29 mm
P. aeruginosa ATCC 9027	20 mm
B. subtilis ATCC 6633	18 mm

Table 9. Antimicrobial Activity as MICS (µg/ml) of tested samples against pathogenic bacteria

Tested microorganisms / samples	Amoxicillin	AgNPs	Combination
***Gram positive bacteria**			
Staphylococcus aureus (RCMB 010028)	3.9 *µg/ml*	62.5 *µg/ml*	3.9 *µg/ml*
Bacillus subtilis (RCMB 010067)	1.95 *µg/ml*	15.63*µg/ml*	0.98 *µg/ml*
Streptococcus pyogenes (RCMB 010015)	3.9 *µg/ml*	62.5 *µg/ml*	7.81 *µg/ml*
Enterococcus faecalis (RCMB 010075)	3.9 *µg/ml*	7.81 *µg/ml*	0.49 *µg/ml*
***Gram negative bacteria**			
Pseudomonas aeruginosa (RCMB 010043)	62.5*µg/ml*	Negative	62.5 *µg/ml*
Escherichia coli (RCMB 010052)	31.25 *µg/ml*	125 *µg/ml*	7.81 *µg/ml*
Klebsiella pneumonia (RCMB 010093)	1.95 *µg/ml*	15.63 *µg/ml*	0.12 *µg/ml*
Proteous mirabilis (RCMB 010085)	62.5 *µg/ml*	125 *µg/ml*	7.81 *µg/ml*

In Table 9 we noted that the O.D of the tested Amoxicillin, AgNPs and their combination indicated Visually by the last test tube have a turbidity in the bacterial growth which means the MIC of tested Amoxicillin, AgNPs and their combination against selected microorganisms whiles the following concentration shows no turbidity and means no growth of microorganisms. Starting with 250*(µg/ml)* of Amoxicillin, AgNPs and their combination and then serial dilution of all takes place until 10^{-12}. Also, Amoxicillin and AgNPs acts as positive control to their combination to explain synergistic phenomenon. In case of gram positive bacteria the Synergistic effect is increase in case of *Bacillus subtilis* and *Enterococcus faecalis* and decrease in case of *Streptococcus pyogenes* while no change in the activity of Amoxicillin after addition of AgNPs in case of *Staphylococcus aureus.* While in case of gram negative bacteria the Synergistic effect is increase in case of *Klebsiella pneumonia* more than *Escherichia coli* and *Proteous mirabilis* which show the same Synergistic effect.While there is no Synergistic effect in case of *Pseudomonas aeruginosa* because of negative effect of AgNPs against it. A synergistic effect of antibiotics Amoxicillin in incorporation with biologically synthesized AgNPs increased the susceptibility among the tested bacteria from 20% - 35**%** [17].The combined effect of AgNPs and Amoxicillin was notably exhibited against *Escherichia coli, Klebsiella pneumonia, Enterococcus faecalis* and *Bacillus subtilis.* These results in line with the findings of [18]. Who mentioned increasing efficacies (percentage) of antibiotics like vancomycin, gentamycin, streptomycin, ampicillin, and kanamycin when used in combination with AgNPs against selected strains.

4. CONCLUSION

In this study, *Bacillus megaterium* was found to be an effective biological tool for the extracellular biosynthesis of stable AgNPs and this method has advantages over other methods as the organism used here is safe. The radiation-induced AgNPs synthesis is a simple, clean and inexpensive process which involves radiolysis of aqueous solution that provides an efficient method to reduce metal ions and further incorporated with the present antibiotics. The Synthesized AgNPs exhibit remarkable antimicrobial activity against both Gram-positive and Gram-negative bacterial strains regardless of their drug-resistant mechanisms. The bactericidal activity have proved that AgNPs kill bacteria at such low concentrations (units of ppm), which do not reveal acute toxic effects on human cell, in addition to overcoming resistance, and lowering cost when compared to conventional antibiotics. It is worth mentioning that this work is a preliminary study and need further brief research and the application for experimental animals before using these data work in application in human health. We hope to use this augmented drug using nanotechnology as topical pharmaceutical. The RSM allowed for the investigation of factor interaction as well as

greatly reducing the number of experiments needed to determine factor significance on nanoparticle formation, thereby reducing costs and increasing experimental efficiency. The antibacterial activity of amoxicillin was augmented when impregnated with AgNPs as shown in Fig.12. On The basis of results from this study, we can deduce that biologically synthesized AgNPs incorporated with amoxicillin may function by binding to thiol (SH) groups of membrane proteins, phospholipid in plasma membrane, enzymes, and phosphate groups of DNA, and can be efficiently used as antimicrobial agents against different pathogenic bacteria such as *E.coli* as shown in Fig. 13. [17].

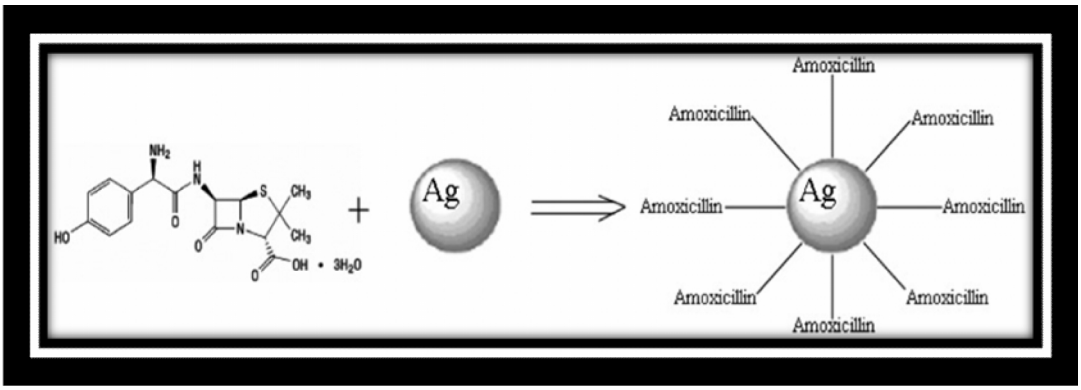

Fig. 12. Illustrated diagram of the structure of amoxicillin and the bonding route for AgNPs chelated with amoxicillin [4]

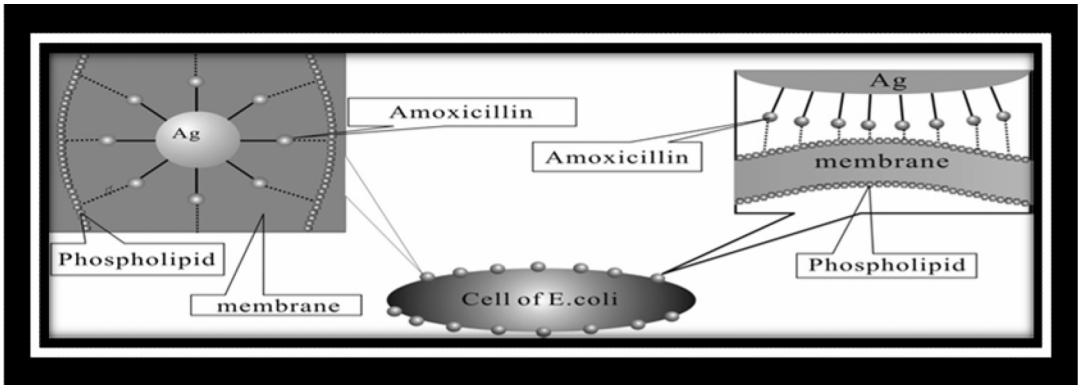

Fig. 13. Illustrated diagram of the combination of AgNPs and amoxicillin reacted with cells extracellularly and intercellularly [4]

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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