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Synergistic effect of biosynthesized silver nanoparticles with antibiotics against multi-drug resistance bacteria isolated from children with diarrhoea under five years

Shahad M.Tawfeeq[1*], Mohammed N. Maaroof [2], Israa Al-Ogaidi [3]

[1*] Department of Biology, College of Science, Tikrit University, Tikrit, Iraq.

[2] Department of Biology, College of Education for Pure science, Tikrit University, Tikrit, Iraq.

[3]Department of Biotechnology, College of Science, University of Baghdad , Baghdad, Iraq.

Abstract

Isolation and identification of bacterial isolates were carried out according to the morphology and biochemical characteristics on one hundred and twenty stool specimens collected from children under five years old via using biochemical tests and Api 20E compact system for further confirmation. Bacterial isolates were distributed as (34.48, 20.68, 5.17,0.86) % for *Escherichia coli, Salmonella typhi* ,*Enterobacter aerogenos, Citrobacter freundii* and *Hafnia alvei* respectively and 9.48 % for each *Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella Pneumonia.* As well as, 2.58% for both *Shigella sonnei* and *Serratia marcescens.* Antibiotic susceptibility test for 116 bacterial isolates was performed towards 20 antibiotics types using disk diffusion method. The results showed dissimilar resistance values towards different antibiotics, ten bacterial isolates were collected for each bacterial species to study their resistance values, the ones with the highest resistance level were selected for further study. Meanwhile, easy and cheap green method using the banana peel extract (BPE) was applied to synthesize silver nanoparticles (AgNPs). Phytochemicals of BPE were screened by standard methods. The results verified the existence of alkaloids, flavonoids, and glycosides in it. These components were act as a reducing agent ,stabilizing and capping agents for AgNO3 with the assistance of the microwave. The successfully preparation of AgNPs was established by ultraviolet-visible spectroscopy, Transmission electron microscopy (TEM), Dynamic light scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR) and zeta potential analysis. The antibacterial activity of the AgNPs against multidrug resistance (MDR) bacteria were studied by using disk diffusion method. The results showed a considerable effect against MDR isolates. The synergistic effects of biosynthesis AgNPs at different concentrations with different standard antibiotic discs (which were Tobramycin, Chloramphenicol, Nitrofuration, Ampicillin-clavulanic acid and Nalidixic acid) against MDR bacteria were also investigated. The result showed the synergistic action of AgNPs and antibiotics leading to enhance antibacterial activity.

Keywords: Silver nanoparticles, Phytochemicals , Antibacterial , Biosynthesis , Synergistic effects.

*Email:Abdbio13@gmail.com

التأثير التأزري لدقائق الفضة النانوية المصنعة حيويا مع المضادات الحيوية ضد البكتريا متعددة المقاومة للعقار والمعزولة من الأطفال المصابين بالإسهال بعمر اقل من خمس سنوات

شهد معاد توفيق[1]، محمد نظير معروف [2]،اسراء العكيدي[3]

[1]قسم علوم حياة، كلية العلوم، جامعة تكريت، تكريت، العراق.

[2]قسم علوم حياة، كلية التربية للعلوم الصرفه، جامعة تكريت، تكريت، العراق.

[3]قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة

جمعت مئة وعشرين عينه براز من الاطفال بعمر اقل من خمس سنوات. وقد تم عزل وتشخيص هذه العزلات البكتيرية بالاعتماد على الشكل الظاهري والاختبارات الكيموحيويه والتشخيص التأكيدي بأستخدام نظام الApi compact system20.وزعت العزلات البكتيرية بنسبة(٤٨. ٣٤، ٦٨. ٢٠ ،٥.١٧، ٨٦. ٠)لكل من

Hafnia alvei,Enterobacter aerogenos,Citrobacter freundii,Salmonella yphi, Eshreichia coli

9.48% لكل من *Pseudomonas aeruginosa, Klebsiella Pneumonia* وايضا 2.58% لكل من *Shigella sonnei , Serratia marcescens* اجري اختبار فحص الحساسية للمضادات، 116 عزلة بكتيرية تجاه 20 نوع مضاد باستعمال طريقة الاقراص.اظهرت النتائج وجود تغاير في مستوى المقاومة تجاه المضادات البكتيرية المختلفة.لقد تم اختيار عزلة بكتيرية واحده من كل نوع بكتيري ذات مقاومة عالية من عشر عزلات للتجارب اللاحقة.من جانب اخر فقد تم تصنيع دقائق الفضة بنجاح باستعمال قشور الموز بواسطة الطريقة الخضراء السهلة والرخيصة. كذلك وقد تم فحص المواد الكيميائية النباتية photochemical لقشور الموز بالطريقة القياسية اظهرت النتائج وجود القلويدات، والفلافونيد، والكلايكوسيدات بمساعدة المايكروويف كعوامل اختزال واستقرار وتغطية لنترات الفضة تم تأكيد بناء دقائق الفضة النانوية بواسطةultraviolet-visible spectroscopy ، electron microscopy Transmission تم اختبار الفعالية Fourier Transform Infrared Spectroscopy (FTIR) , Dynamic light Scattering و zeta potential ضد البكتريا متعددة المقاومة ضد البكترية لدقائق الفضه النانوية للمضادات الحيوية باستخدام طريقة نشر الاقراص اذ ظهرت النتائج تأثير جيد ضد العزلات متعددة المقاومة للعقار.وكذلك فقد اختبر التأثير التأزري لدقائق الفضة بتراكيز مختلفة مع انواع مختلفة من اقراص المضادات (التي كانت Nitrofuration ،clavulanic acid, Chloramphenicol–Nalidixic acid, Ampicillin ،Tobramycin) ضد البكتريا متعددة المقاومة المضادات الحيوية . لقد اظهرت النتائج بأن التأثير التأزري لكل من دقائق الفضة واقراص المضادات الحيوية ادى الى تعزيز الفعالية المضادة للبكتريا .

Introduction

The diarrheal disease is one of the main reason of childhood mortality and morbidity in the developing countries [1].It is consider a top of the killers for the children under five years old [2]. The diarrheal disease can be classified into three main clinical kinds: acute watery, persistent and bloody diarrhea [3].The diarrhea happens as a result of the entry of pathogens to intestinal cavity of children through food, water or hands which can be contaminated by the pathogens , or as a result to transforming some members of the normal flora, when the changes happened in the intestinal environment as a result of taking a particular drug or via infecting the child with pathogenic bacteria [4,5]. The principal microorganisms which implicated in diarrhea disease belong to the enterobacteriaceae. These family are gram-negative rods, facultative anaerobes or aerobes, possess a complex antigenic structure [6]. Most of these isolates are resistant to different antimicrobial agents for example the carbapenems, which are often claimed to be "the last line of antibiotic defense" against resistant microorganisms [7].Although many of new antibacterial agents were developed in the last few decades none of them have improved its activity against multidrug-resistant bacteria [8,9].Recently, nanotechnology has important in the pharmaceutical and biomedical areas as

alternative antibacterial strategy due to re-emergence the appearance and infectious diseases of antibacterial-resistant strains particularly within gram negative bacteria[10]. The metallic nanoparticles are the most promising material as antibacterial activity, and it gain the current interest in research due to the growing microbial resistance against antibiotics and the developing of the resistant strains [11]. AgNPs have shown antibacterial activity against a wide range of microbes, probably via their multiple mechanisms of antibacterial action [12]. AgNPs can be synthesized by several physical, chemical and biological methods. Where the green synthesis method is one of such promising processes because of avoiding toxicity of the process and by increasing the quality of the production that made it replaced the chemical methods [13]. In the biological method (green method) employing a natural reducing agent such as plant extract, enzyme, microorganism, polysaccharide [14]. Meanwhile, the conjugation between silver nanopaerticles and antibiotics such as; penicillin G, vancomycin, amoxicillin and erythromycin drove to enhance and synergistic the antibacterial impacts against Gram-negative and Gram-positive bacteria [15]. Hence, the synergistic action will permit to use low concentration of AgNPs that earnings it has low toxicity to humans with accepted dose of antibiotics [16]. Therefore, enhancing the synergistic effect of bio-synthesized AgNPs and antibiotics against multi drug resistance (MDRs) bacteria could be potentially apply in the developing of new therapeutic agents[17,18,19]. This study aimed to isolate and diagnose some bacterial pathogens which caused diarrheal disease. In addition, synthesize AgNPs by biological method by using the extract of banana peel waste and characterize the synthesized NPs by utilizing UV-vis ,TEM , zeta potential, DLS and FT-IR analysis. Besides, their antimicrobial activity against multi-drug resistance bacteria were tested. As well as, we investigated the synergistic activity of several types of antibiotics discs (which were Tobramycin, Chloramphenicol, Nitrofuration, Ampicillin- clavulanic acid and Nalidixic acid) with different concentrations of AgNPs against multidrug resistance isolates which were *E.coli, P.mirabilis, S.typhi* and *P.aurignosa*,.

Materials and Methods

Isolation and identification of clinical isolate

A total of 120 stool sample were collected in a disposable plastic containers from patients with diarrhea (infants and children under five years old) in different wards of Iraqi hospitals, during the period from May 2015 until October 2015, the relevant information were recorded from every patient included; age (1-5 years),sex and other information. The samples were transferred to the lab for direct macroscopical examination and as well microscopical examination. The samples was cultured on MacConkey and blood agar and incubated at 37°C for 24 h. Thereafter the growing colonies were refined on differential and selective media. After distinguish by depending on morphological and biochemical tests such as oxidase test, Catalase test, and IMViC according to the identification scheme described by[20], then was further confirmed by API 20 E.

Antibiotic susceptibility testing by disk diffusion method

For antimicrobial susceptibility test using the McFarland standard solution which used to standardize the inoculum density. This test determined by the disk diffusion method on Muller-Hinton agar following the Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. In this test a sterile swabs were used to inoculate the suspension after compared with McFarland standard solution by streaking 0.1 on the mueller hinton agar plate. It was then allowed in room temperature for 5 minutes. Sterile forceps were used to place five antimicrobial discs in every plate. Thereafter the plate was incubated at 37°C for 18-24 hrs. Results were recorded and compared with the standard levels to CLSI documentation [21].

Preparation of silver nanoparticles

The silver nanoparticles (AgNPs) were prepared by biological method according to [22] with slight modification ; which include the use of microwave to shorten the time as well as to preserve the vital resources in the banana peels .

Preparation of Banana peel extract (BPE)

The method involved is as follows; 100 gm of banan peels was cleaned and cut into pieces and taken into 100 ml of distilled water. Then the solution was heated in microwave for 2 min. After that the solution was filtered through a cheese cloth to remove insoluble fractions and macromolecules. Filtered solution was treated with equal volume of chilled acetone and the precipitate was centrifuged at 1000 rpm for 5 min. This precipitate was resuspended in distilled water and stored at 4^{o} C for further studies. This extract was used as reducing as well as stabilizing agent.

Preparation of Ag nanoparticles by using the extract of BPE

Silver nitrate ($AgNO_3$) in distilled water was the source of silver in this study. Typical reaction mixtures contained 1 ml of BPE (equivalent to 6.8 mg dry weight) in 49 ml of silver nitrate solution (1 mM) unless otherwise stated. The reaction mixture was incubated in microwave for 1 minute. Finally yellowish brown colour was appeared indicating the presence of Ag nanoparticles.

Characterization of synthesized silver nanoparticles

The UV-Visible spectra of silver nanoparticles were recorded as a function of wavelength using UV-Vis spectrophotometer operated at a resolution of 1 nm. This is a simple method that give information about particle concentration and size, and size/size distribution, the shift of absorbance relay on the size (diameters) and shape of particles [23].The shape and size of silver nanoparticles were determined by TEM. For TEM, a drop of aqueous silver nanoparticles sample was loaded on a carboncoated copper grid, and it was allowed to dry in room temperature, the micrographs were obtained using TEM. The average particle size determined by DLS whereas, The surface charge and particle size distribution of silver nanoparticles determine by Zeta potential.Fourier Transform Infrared Spectroscopy (FTIR) measurements were carried out using infra-red spectrometer by employing KBr pellet technique. The functional groups which present in biomolecules in the plant extract where detection by using the FTIR Spectrometer.

Antimicrobial activity of synthesized silver nanoparticles

The antibacterial efficacy of the phytosynthesized AgNPs was investigated by agar well diffusion assay[24], against various types of multidrug resistant bacteria isolated from clinical samples. The tested microorganisms included; *E.coli , P.aeruginosa, K.pneumonia, P.merabilius, E.aerogenes , S.marcescens* and *S.typhi* approximately (108 colony-forming units/mL) were swabbed uniformly on Mueller hinton agar plates employing sterile cotton swab, then, four wells of 6-mm diameter were made employing sterile well borer. Fifty microliter of AgNPs solutions was poured into the corresponding well.. Control sample (BPE) was employed to assess the antimicrobial activity of BPE. The plates were then incubated at 37° C for 24 hrs and diameter of inhibition zone was measured.

Disk diffusion assay to evaluate synergistic effect

The synergistic effects of green synthesized silver nanoparticles with various antibiotics for bactericidal activity were studied according to [43] against MDR isolates on Mueller hinton agar plates using disk diffusion method. The standard antibiotic discs used were Tobramycin, Chloramphenicol, Nitrofuration, Ampicillin-clavulanic acid and Nalidixic acid. The inocula were prepared by diluting in 5 ml of NaCl which compared with 0.5McFarland standard and the spreading on the plate. Five microliters of different concentration (15 , 30 , 60)Mg/ml for AgNPs were added to the discs. Then the plates were placed and kept for incubation at 37° c. After 24 hours, the inhibition zone were measured. [15], and compared with Standard antibiotics which tested in Antibiotic susceptibility.

Results & discussion

Isolation and identification

Out of 120 stool samples that cultured, only 4 cases have no bacterial growth. These cases may attributed to many reasons, like unrecognized agents such as anaerobic or microaerophilic bacteria; viral infection were excluded from our study. Isolation and identification of 116 isolates by biochemical tests and API showed that (34.48%) 40 isolates were *E. coli,* (20.68%) 24 isolates were *Salmonella typhi,* (9.48%), 11 isolates were *Psedomonas aeruginosa* ,(9.48)11 isolates were *Klebsiella Pneumonia* , (9.48) 11 isolates were *Proteus mirabilis,* (5.17%) 6 isolates were *Citrobacter freundii,* (5.17%) 6 isolates were *Enterobacter aerogenes,* (2.58%) 3 isolates were *Shigella sonnei* (2.58%) 3 isolates were *Serratia marcescens* and (0.86) 1 isolates were *Hafnia alvei.*

Antimicrobial susceptibility test

The Majority of the isolates showed multidrug resistance profiles where it has been found that (100%) of isolates were resistant to Mitronidazole and Vancomycin. Whereas, the resistance was(98.3%) for each of the Penicillin G and Clindamicin, (97.41%) to Erythromycin, (96.55%) to Refampin, (62.93%) to Cefotaxim, (60.34%) to Pipracillin, (59.48%) to Trimethoprim/sulfamethoxazole, (57.75%) to tetracycline, (56.89%) to Ampicillin-sulbactam, (50.86%) to Amoxicillin-Clvulanic, (50%) to Nalidixic acid, (43.1%) to Tobramycin, (34.48%) to Nitrofuration, (30.8%) to Gentamicin, (25.86%) to chloramphenicol, (25%) to Ciprofloxacin, (11.2%)

to Amikacin and (0.86%) to Imipenem as illustrated in Figure -1. Recording to the results of the current study, most bacterial isolates were possessed resistance to beta-lactam antibiotics, this is due to the ability of bacteria to produce B-lactamase enzymes [25].Therefore, the mean reason for choosing Tobramycin, Chloramphenicol, Nitrofuration, Ampicillin-clavulanic acid and Nalidixic acid to be under study because of the high resistance of some isolates to these antibiotics and the core goal of this work was to reactivate these antibiotics efficacy and reduce its microbial resistance through conjugation with silver nanoparticles.

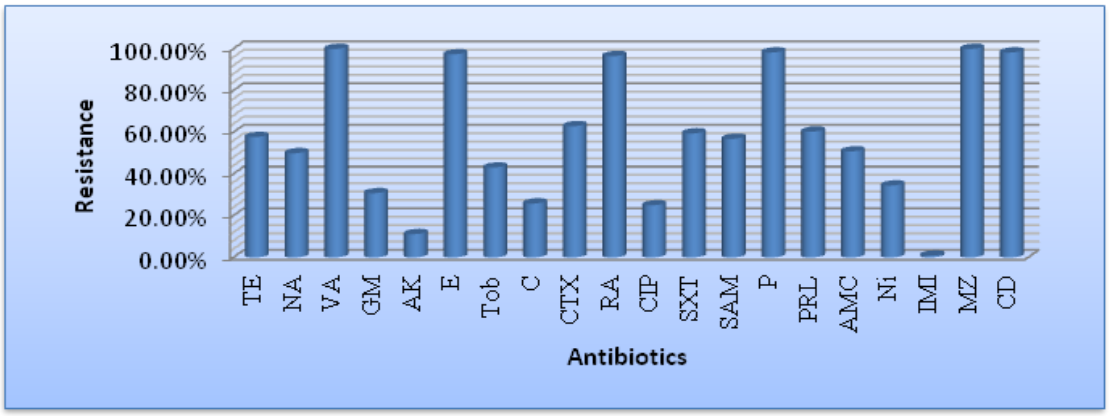

Figure 1- Antibiotics susceptibility against resistant bacteria **TE**: Tetracycline; **NA**: Nalidixic acid; **VA:** Vancomycin; **GM:** Gentamicin; **AK:** Amikacin ; **E:** Erythromycin ; **TOB:** Tobramycim ; **C:** Chloramphenicol ; **CTX:** Cefotaxim; **RA:** Refampin ; **CIP:** Ciprofloxacin ; **SXT:** Trimethoprim/sulfamethoxazole ; **SAM:** Ampicillin-sulbactam ; **P:** Penicillin G; **PRL:** Pipracillin ; **AMC:** Amoxicillin-Clvulanic; **NI:** Nitrofuration; **IMI:** Imipenem ; **MZ:** Mitronidazole; **CD:** Clindamicin.

Synthesis and Characterization of Ag nanoparticles by Banana pell extraction (BPE) and microwave

Visualization of color:

In this research, the extract of banana peel was used as a stabilizer and appropriate polymeric media for reducing the $AgNO_3$. After 2 min of mixing the BPE with aqueous solution of the $AgNO_3$ the colourless solution turned to yellowish brown which indicating the generation of Ag nanoparticles, that means the active molecules present in the BPE caused the reduction of silver metal ions into silver nanoparticles [22]. This characteristic difference in colour attributed to the excitation of SPR in the metal NPs [26]. whereas no change in colour by $AgNO_3$ solution (control) was observed. For the synthesis of AgNPs a microwave-assisted method [27,28] was used. The chemistry of microwave involves a dipolar mechanism and ionic conduction [29,15]. This method makes reaction faster and obtain a higher yields of AgNPs with the same exposure and temperature [30]. In addition, the synthesis by microwave requires lower energy consumption in comparison with conventional heating method. Figure -2 describes the development of the color during preparing silver nanoparticles.

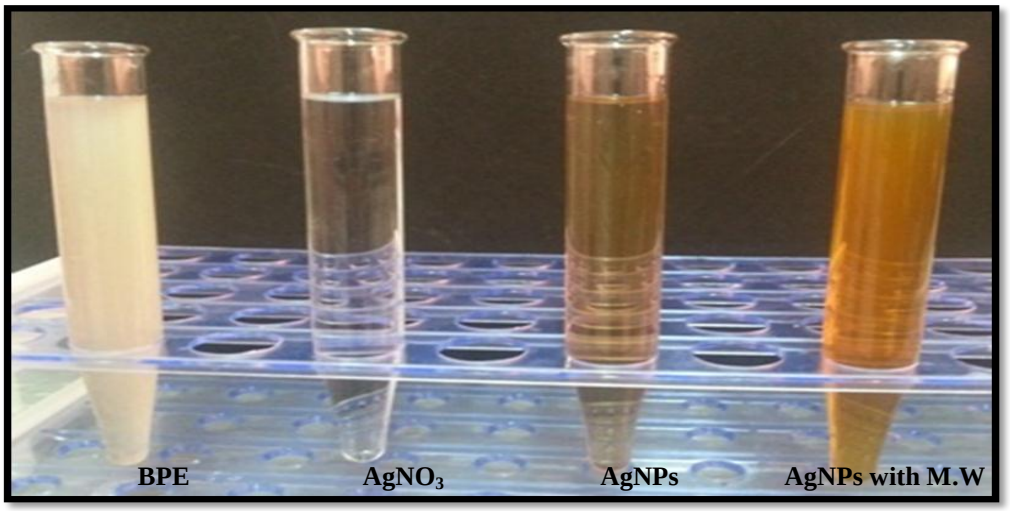

Figure 2- shows steps of AgNPs preparation by using microwave

Characterization of silver nanoparticles (AgNPs)

As can be seen in Figure- 3, spectrophotometer measurements of silver nanoparticles (AgNPs) showed a peak at 415 nm belong to the phenomenon of SPR, that happen owing to the excitation of the surface plasmons existed on the outer surface of the AgNPs that get excited owing to the applied electromagnetic field [31]. This result is agree with the result which obtained by [32].

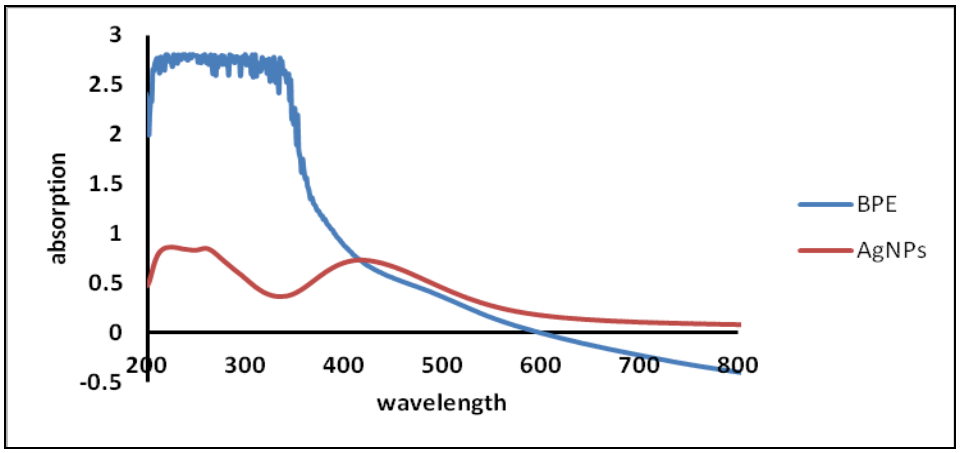

Figure 3- UV-Vis spectrophotometry of silver nanoparticles produced by BPE

The shape and size of the biosynthesized silver nanoparticles were studied by TEM. The images obviously showed that particles size were in the range from 9 to 15 nm Figure- 4 (a). These results revealed that the particles were spherical in shape and uniformly distributed (mono dispersed) without important agglomeration. Another findings in accordance with the measurements of particle size analyzer based on dynamic light scattering technique, showed the particle size distribution of silver nanoparticles with average size 40.5 nm. Also another important finding was zeta potential measurements, Figure -4(b) indicate the stability values of zeta potential at a minimum of ±30 mV of nanosuspension which showed a good stability of the colloidal of AgNPs [33].

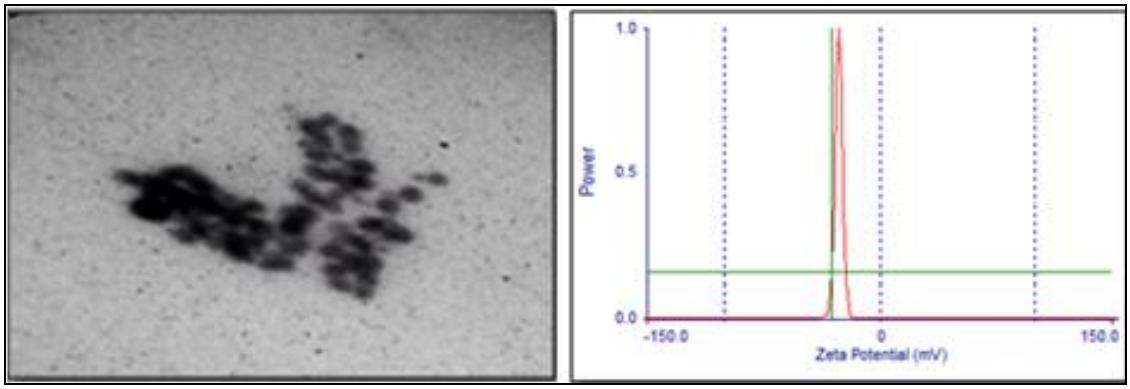

Figure 4- (a) Transmission electron microscopic (TEM) images of the AgNPs. **(b)** Zeta potential for AgNPs.

The FTIR analysis of BPE is presented in Figure -5, various peaks appeared at 3423.12, 2932.6, 1744.26, 1621.35, 1240, 1054.33, 772.63 and 467.62 cm-1 were assigned to stretching vibration of O-H of alcohol or N-H of amines, C-H of alkanes, C=O of carboxylic acid or ester, N-C=O amide I bond of proteins, CH2 of alkanes, C-O of carboxylic acid, ester, or ether, C-N of aliphatic amines or alcohol/phenol, N-H deformation of amines, and C-C bending, respectively [34]. In addition, FT-IR analysis of AgNPs revealed the strong bands at 3436.99, 2923.45, 2362.69, 1626.08, 1378.14, 1063.25, and 776.53 cm-1. Farthermore, the AgNPs showed a broad absorption bands appearing at 3436.99 cm^{-1} is assigned for O−H stretching vibration [35]. Presence of the sharp peak at 2923.45cm^{-1} was assigned to C–H stretching vibration. Also, The sharp and strong absorption band at 1626.08cm^{-1} and 1378.14cm^{-1} assigned to the stretching vibration of N-H and C=O group respectively, where 1063.25 cm^{-1} assigned to C-N stretching[36].

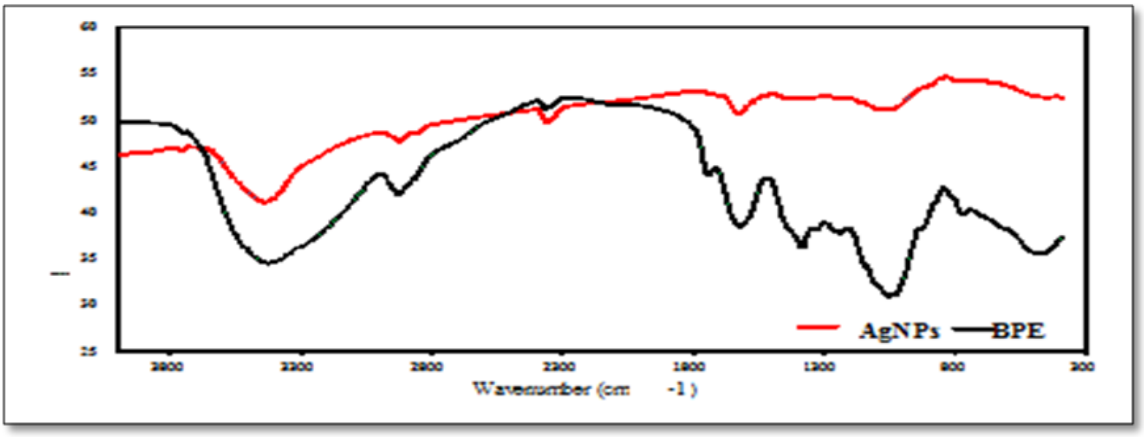

Figure 5- Fourier transform infrared spectroscopy of silver nanoparticles

Antimicrobial activity Assay

Antimicrobial efficacy of silver nanoparticles

The antimicrobial activity of the silver nanoparticles (AgNPs) was examined against studied pathogenic bacteria, Primary screening revealed that the AgNPs showed more antibacterial activity compared with the $AgNO_3$, Table- 1 , where the inhibition zone diameter were (10, 13, 14, 15,16 ,17 mm) for *Proteus mirabilis*, *Serratia marcescens* , *E.coli*, *Enterobacter aerogenes*, *Salmonella typhi*, *Klebsiella pnemoniae* and *Psedomonas aerugenosae*, respectively. The high bactericidal activity of AgNPs which caused by their extremely large surface region, which provides better contact with pathogenic bacteria [22]. There are several mechanism of silver nanoparticles ;the first mechanisms, the impact may be due to ultra fine size for the AgNPs and the larger surface region, while their positively charged Ag+ ions attach to the negatively charged which present in bacterial cell wall, leading to deactivating the cellular enzymes, therefor causing disruptions in the membrane permeability [37]. The second, AgNPs via interactions with the thiol group of L-cysteine protein

residues will lead to enzymatic dysfunction [38]. Finally, the silver nanoparticles causes damage on proteins and DNA via release of reactive oxygen species (ROS) [39].

Table 1 - Antimicrobial activity of silver nitrate, banana pell extract and silver nanoparticles against representative human pathogenic bacterial strains.

Microorganisms	Diameter of inhibition zone (mm)		
	BPE	$AgNO_3$	AgNPs
Proteus mirabilis	Nil	0	10
Serratia marcescens	Nil	0	13
E.coli	Nil	0	14
Enterobacter aerogenes	Nil	0	15
Salmonella typhi	Nil	0	16
Klebsiella pnemoniae	Nil	0	17
Psedomonas aerugenosae	Nil	0	17

Synergistic effect of AgNPs with antibiotics by disk diffusion methods

The synergistic impact of silver nanoparticles and antibiotics leading to enhance antibacterial activity ; therefore, the development of resistance pathogenic bacteria can be treated via the simultaneous action of AgNPs and antibiotics. In addition it would reduce the amount of the administered antibiotic. The bonding reaction between AgNPs and antibiotic may causes increasing the synergistic effect [15]. In this study the synergistic effects of AgNPs with 5 antibiotics against *P. merabilis, P. aeruginosa, S. typhi* and *E. coli* were studied by using disc-diffusion method. The diameter of the inhibition zone increased when tested Tobramycin, Nitrofuration, Nalidixic acid, Ampicillin-Clavolanic acid and Chloramphenicol in the presence of the metallic nanoparticles at different concentrations (15, 30, 60)µg/ml against the tested isolates, the result shown in Table -2, this effect may be come from either increasing the drug bio-availability after conjugation in the cell membrane of bacteria or may be by assimilatory effect of both components [40-42]. Therefore, the use of AgNPs in the association with antibiotic showed synergistic effect. Previous studies proposed that the AgNPs could work in two ways the first one; it can attack the cell membrane to destabilize it, the second way ; the AgNPs- antibiotic can easily a crosses the cell membrane barrier and show their bioactivity [40-42].This study describes a promising strategy to develop new antibiotic substance against the multidrug resistance bacteria. It can help to develop a new antibiotic to fight the threat which posed via the evolution of new antibiotic resistance mechanisms of the MDR bacteria.

Table 2 -The inhibition zone of silver nanoparticles with and without different types of antibiotics against multidrug resistance bacteria

Organism	The concentration of silver Mg /ml	control	Zone of inhibition (mm)									
			Standard Chloram	sample	Standard Tobra	sample	Standard Nitrofuran	sample	Standard Amp+ sulbac	sample	Standard Nalidixic	sample
P. merabilis	15	Nil	18	20	15	12	17	18	15	17	19	31
	30			20		15		19		16		32
	60			23		12		22		16		30
P. aeruginosa	15	Nil	18	10	15	18	17	22	15	18	19	29
	30			21		14		21		16		30
	60			22		14		23		18		28
S. typhi	15	Nil	18	20	15	14	17	16	15	22	19	17
	30			11		19		17		23		16
	60			11		20		17		22		16
E. coli	15	Nil	18	22	15	15	17	27	15	10	19	16
	30			23		16		26		11		16
	60			23		14		22		10		17

Conclusion

Banana peels as agricultural waste material was successfully utilized for the consistent and quick synthesis of silver nanoparticles and would be appropriate for emerging a biological method for large-scale production. Synthesized silver nanoparticles revealed good antimicrobial activity (in vitro) against the selected pathogenic microorganisms.the study emphasized on a possible combination of antibiotics (Tobramycin, Chloramphenicol, Nitrofuration, Ampicillin- clavulanic acid and Nalidixic acid) with Ag NPs, which showed enhanced antimicrobial effects and was concluded as synergism. In this context, synergistic antibacterial property of silver nanoparticles with these antibiotics, is considered as an alternative and attractive method to combat the increasing spread of drug resistance and such an approach is likely to provide much potential application in medical devices and microbial resistant system. Since these antibiotics are relatively costly, in our study we reduce the concentration of those antibiotics for minimizing the cost and side effects by combining them with silver nanoparticles.

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