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The Antimicrobial Activity of Melanin-Mediated Synthesis of Silver Nanoparticles

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ABSTRACT

Background: Nowadays, the environmentally friendly procedures must be developed to avoid using harmful compounds in synthesis methods. Their increase interest in creating and researching silver nanoparticles (AgNPs) because of their numerous applications in many fields especially medical fields such as burn, wound healing, dental and bone implants, antibacterial, viral, fungal, and arthropodal activities. Biosynthesis of nanoparticles mediated pigments have been widely used as antimicrobial agent against microorganisms. Silver nanoparticles had synthesized by using melanin from locally isolate *Pseudomonas aeruginosa*, and used as antimicrobial activity against pathogenic microorganisms. **Aim of the study**: Isolation of *Pseudomonas aeruginosa* that produce melanin and extraction of melanin. Synthesis and characterization silver nanoparticle and study of the antimicrobial activity of silver nanoparticles in the presence of melanin against UTI pathogens.

Materials and methods: The samples swab inoculated on cetrimide agar as selective media and incubated aerobically for 24 hours at 37 °C. Used nutrient agar with nutrient broth supplement with 1% tyrosine for screening for melanin production by *P. aeruginosa* isolates, silver nanoparticles synthesis from *P. aeruginosa* was done according to biological method and was characterized with AFM, UV-Visible, XRD, FTIR and FE-SEM. Agar well diffusion method was used to examine the effect of combination against UTI pathogens.

Results: The synergistic effects of AgNPs and melanin were evaluated to compare between the two treatments (silver nanoparticles alone and combination of silver nanoparticles and melanin). The results revealed that the combination showed the highest antimicrobial activity in compare with silver nanoparticles alone.

Keywords: *P. aeruginosa*, detection, melanin pigment, characterization AgNPs, Synergistic effects, (FE-SEM), (FTIR), XRD, AFM.

INTRODUCTION

A major worry is the rise of bacteria that are resistant to antibiotics in the medical field and microbes evolved a number of strategies during molecular evolution to maintain genomic flexibility for example horizontal gene transfer, enzyme promiscuity, quorum sensing and biofilm formation, this genomic adaptability creates adequate environment for growth and survival when exposed to harsh environments [(1)].

As a result of increased appearance infectious diseases and the emergence of antibiotic-resistant strains, particularly Gram-negative bacteria [(2)].

As human fluids body contain high levels of sulfide and chloride ions, they defend against silver toxicity by forming insoluble salts containing silver ions, so for this reason silver has a relatively low toxicity in humans [(3)]. The mechanism way for AgNPs for antibacterial activity is demonstrated by anchoring to and penetrating bacterial cell walls, as well as cellular signaling modulation [(4)]. When the nanoparticles are formed, they prefer to be stabilized before they can be used so several reagents have been reported to act as stabilizing agents [(5)]. Melanin biopolymer may act as a reducing and stabilizing mediator in the formation of silver nanostructures [(6)].

MATERIALS AND METHODS

Collection and Identification of *P. aeruginosa* isolate

The samples were clinically isolated from Iraqi patient's hospitals (burns, UTI, tracheostomy patients in ICU, endotracheal tube patients in ICU, mastoiditis, and wound infection). All samples swab inoculated on cetrimide agar as selective media and incubated aerobically for 24 hours at 37 °C. Isolates were initial identification by cultural characteristics, biochemical tests, and Vitek -2 system.

Screening for melanin production by *P. aeruginosa* isolates

The bacterial strains were inoculated and incubated on cetrimide agar at 37°C for 24 hr. After that, a loopful of bacterial strains was inoculated to nutrient agar and nutrient broth supplement with 1% tyrosine to detect the ability of bacteria strains to produce melanin. This method was described clearly by **Surwase *et al.*** [(7)].

Production of melanin

Liquid medium was used for *P. aeruginosa* cultivation and melanin production according to **Surwase *et al.*** [(7)].

1- Nutrient broth was dissolved in150 ml of D.W. (500ml Erlenmeyer flask) supplement with 1% of tyrosine
2- The pH has been fixed at 7.0
3- The medium was sterilized by autoclave for 15 minutes at 15 lbs pressure (121°C).
4- After sterilizing, kept it for cooling, a colony of fresh culture was added to this medium and placed in a rotary shaker moving incubated at 37°C 160 rpm for 3 to 6 days until the liquid medium became darkly pigmented.

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Extraction and purification of melanin pigment

The highest-produced isolate was used to produce melanin, as mentioned by **Roy & Rhim** [8] by following steps:

1. To separate the supernatant from cells and debris, for 15 minutes, the medium was centrifuged at 8000 rpm
2. Chloroform was mixed with the above solution to deproteinize the melanin pigment, each tube (10) ml add (2) ml chloroform
3. To ensure complete polymerization of melanin, 5M of NaOH was used to adjust pH to 10 and further autoclaved at 120°C for 20 min [9].
4. After autoclaved solution completed centrifuging the solution at 5000 g for 5 min and the supernatant was collected.
5. 5M HCl was added carefully to acidify the solution to pH 2 until melanin precipitate
6. After the melanin precipitate by centrifuged for 20 min at 8000 rpm to extract crude melanin, and then equal volumes of chloroform, ethyl acetate, and double methanol (1V:1V:2V) were added and combined.
7. To obtain melanin dissolved in this mixture, for 15 min, the mixture was centrifuged at 8000 rpm, and then put in a glass Petri dish until solvent evaporation, 2 to 3 times this step was repeated to have powder melanin once dried, the purified preparation was kept at room temperature.

Synthesis of Silver nanoparticles (AgNPs)

After isolation and identification of *P. aeruginosa* inoculum was prepared by culturing a colony from an agar plate with a loop and aseptically transferred into a 100 ml brine heart infusion broth medium dissolved in deionized water. the medium was cultured at 37°C and 180 rpm for 2 days in order to prepare a suspension of the *P. aeruginosa* bacterium. after that, the supernatant centrifuged for 10 min at 8000 rpm. The supernatants (free of any kind of precipitates) were passed through sterilized membranes of 0.2µm pore diameter before being used as catalysts for AgNPs synthesis.

Four gm of AgNO3 was added to this suspension and incubated in dark environment at 37°C under agitation at 120 rpm for for 24 h, the color of the reaction mixture changed to brown which indicates the formation of silver nanoparticles. Then the mixture centrifuged at 8000 rpm for 10 min. The supernatant was removed, and precipitate was washed with deionized water and centrifuge at 8000 rpm for 5 min and put it in glass petri dish until evaporation to have powder AgNPs once dried according to **Elbeshehy *et al.*** [10].

Characterization of silver nanoparticles

physical and Morphological characterization of synthesis silver nanoparticles was done. UV-Visible Spectroscopy analysis [11], (FE-SEM) Field emission scanning electron microscopy [12], X-Ray Diffraction method analysis [13], Fourier transform infrared spectroscopy (FTIR) analysis [14], and (AFM) Atomic force microscopy [15].

Antimicrobial activity of the extract melanin and silver nanoparticles by using well-diffusion assay (WDA)

AgNPs and pigment were used to examine their antimicrobial activity against 7 human clinical isolates pathogen, *Staphylococcus aureus*, *Staphylococcus Haemolyticus*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis*, *Acinetobacter baumannii* and yeast *Candida albicans*.

Sterile cotton swabs were used to transfer and spread the test microorganism onto the agar medium, and then 5 wells were made into the agar using a sterile egel puncture with a diameter of 4 mm. After that, various concentrations (8,16,32,64, 128, 256, 512 and 1024 µg/ml) of AgNPs, extract melanin, and a combination of both were added to the wells and then incubated for 24 hrs at 37 °C. AgNPs were dissolved by an ultrasonic cleaner device in deionized sterile water. After incubation, the inhibition zones were measured in millimeters to determine the antimicrobial activity of extract melanin and silver nanoparticles and combination [16].

Ethical approval

This study was approved by the Ethical Committee, Department of Biology, College of Science, University of Bagdad, Baghdad, Iraq and the Iraqi Ministry of Health. Ref.: CSEC/0122/0033.

Statistical Analysis

Statistical analyses were performed by using SPSS software version 25.0 (SPSS, Chicago). Continuous data were presented as mean and standard deviation and analyzed with Student t-test. Categorical variables were expressed as number and percentage and analyzed with Chi-square test. Receiver operating characteristic curve (ROC) was used to evaluate the predictive value of different markers in prediction of different complications and outcome. A p- value less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

Isolation and Identification of *Pseudomonas aeruginosa*

One hundred and nine clinical samples were collected from different sources (wound infection, burns patients, UTI, tracheostomy patients in ICU, endotracheal tube patients in ICU and mastoiditis) as showing in the table **1.**

Table 1: Number and percentage of *Pseudomonas aeruginosa* isolates with specimens' source

Source of Samples	Number of Specimens	Number of *P. aeruginosa*	Number (%) of *P. aeruginosa*
Wound infection	**23**	**7**	**30.43%**
Burns	**28**	**15**	**53.57%**
UTI	**14**	**2**	**14.28%**
Tracheostomy patients in ICU	**21**	**5**	**23.8%**
Endotracheal tube patients in ICU	**18**	**4**	**22.22%**
Mastoiditis	**5**	**2**	**40%**

The preliminary identification of *P. aeruginosa* was carried out on MacConkey agar they appear as pale colony (lactose non-fermenter) and on nutrient agar isolates were able to grow at 42°C and has sweat grape odor. *P. aeruginosa* is distinguished from other *Pseudomonas* species by its capacity to grow at high temperatures and some isolates have ability to produce pyocyanin pigment they were able to grow on cetrimide agar is a selective/differential medium because it contains 0.03% cetrimide that prevents the growth of microorganisms Biochemical tests showed that all the 35 isolates were positive for oxidase, urease and catalase tests, oxidase test and positive urease production test The results of IMViC tests showed that all isolates were indole, methyl red (MR) and voges-proskauer (VP) negative and had able to utilize citrate as a sole carbon source.

Screening for melanin producing *P. aeruginosa*

From 35 strains of *P. aeruginosa* only four isolates which are identifications by Vitek -2 system had the ability to produce a brown - black diffusible pigment (Melanin) as showing in the figure **1**.

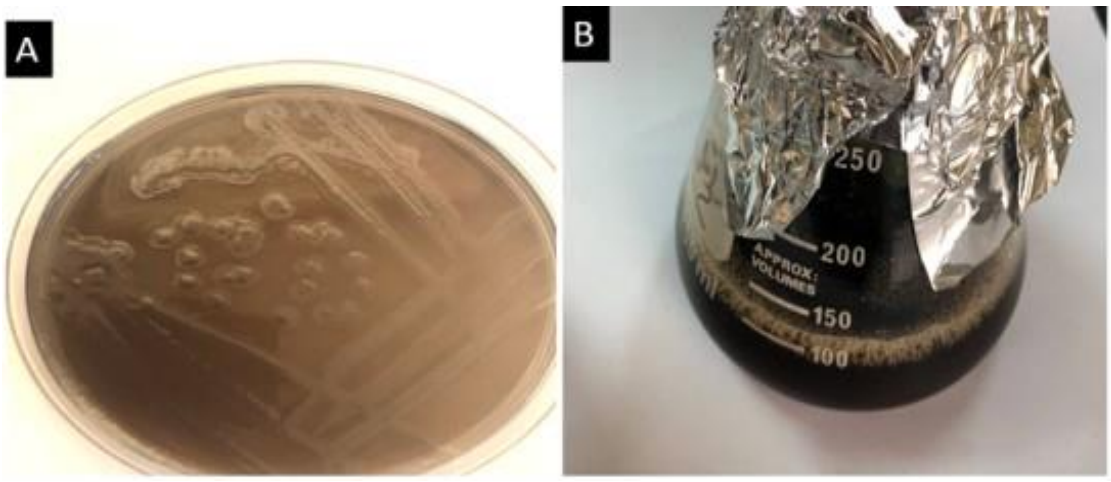

Figure 1: Melanin pigment production of *P. aeruginosa* (A) on nutrient agar supplement with L-tyrosine (B) on nutrient broth supplement with L-tyrosine؛

Extraction and purification of *P. aeruginosa* melanin pigment

The extraction was performed as described by [14] with slight modifications as shown in figure **2**. many steps involve to having melanin powder these steps are illustrated in discussion section.

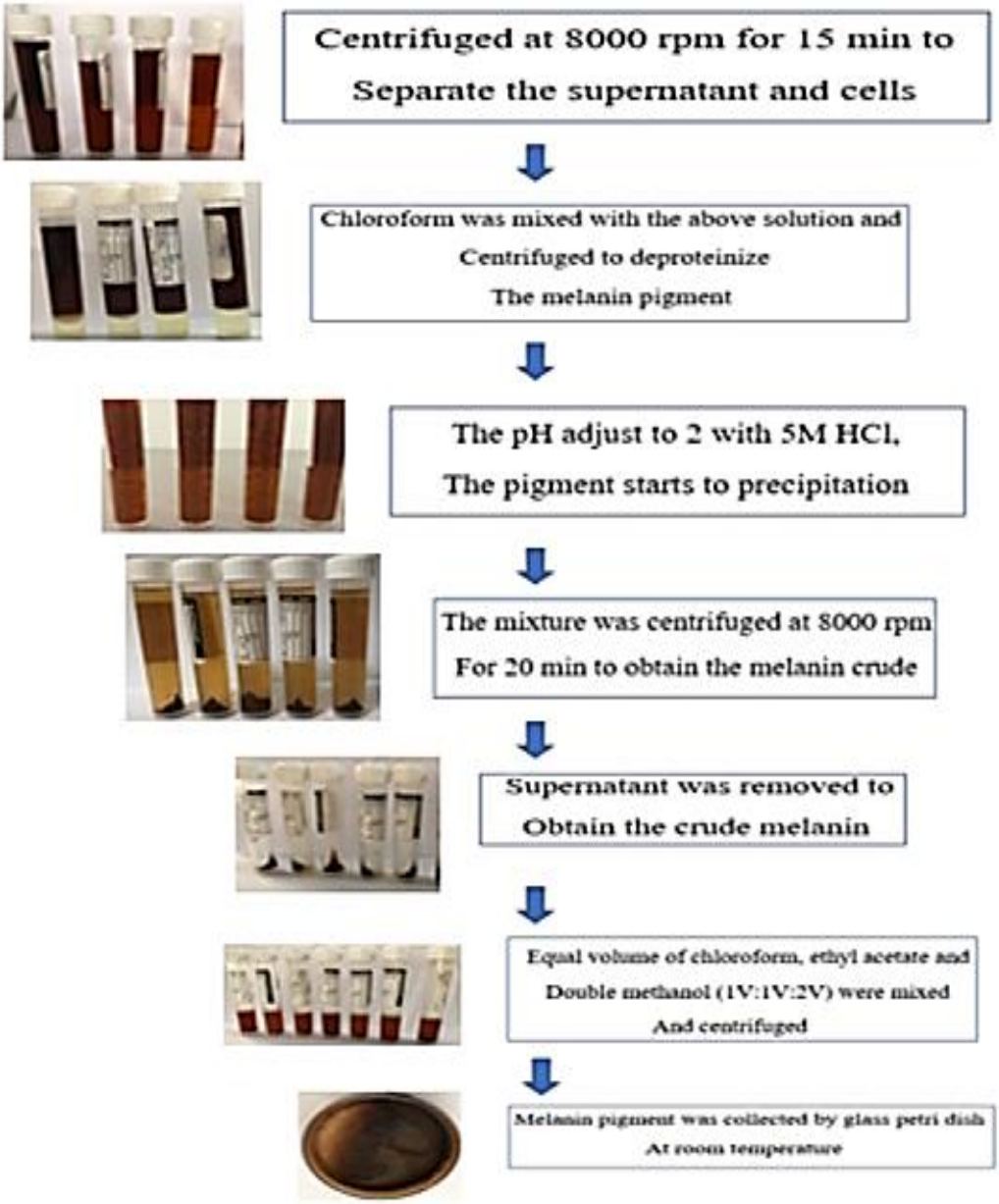

Figure 2: Extraction steps of melanin pigment

Characterization of silver nanoparticles

Atomic force microscopy (AFM) analysis

The atomic force microscopy was used as a confirmatory technique to characterize the biosynthesis of AgNPs by detecting their average diameter in addition to the morphology of a nanoparticle's surface in two and three dimensions down to the atomic level. The results obtained in this study showed that the biosynthesized AgNPs by *P. aeruginosa* had average diameter of (46.17) nm as shown in figure **3.**

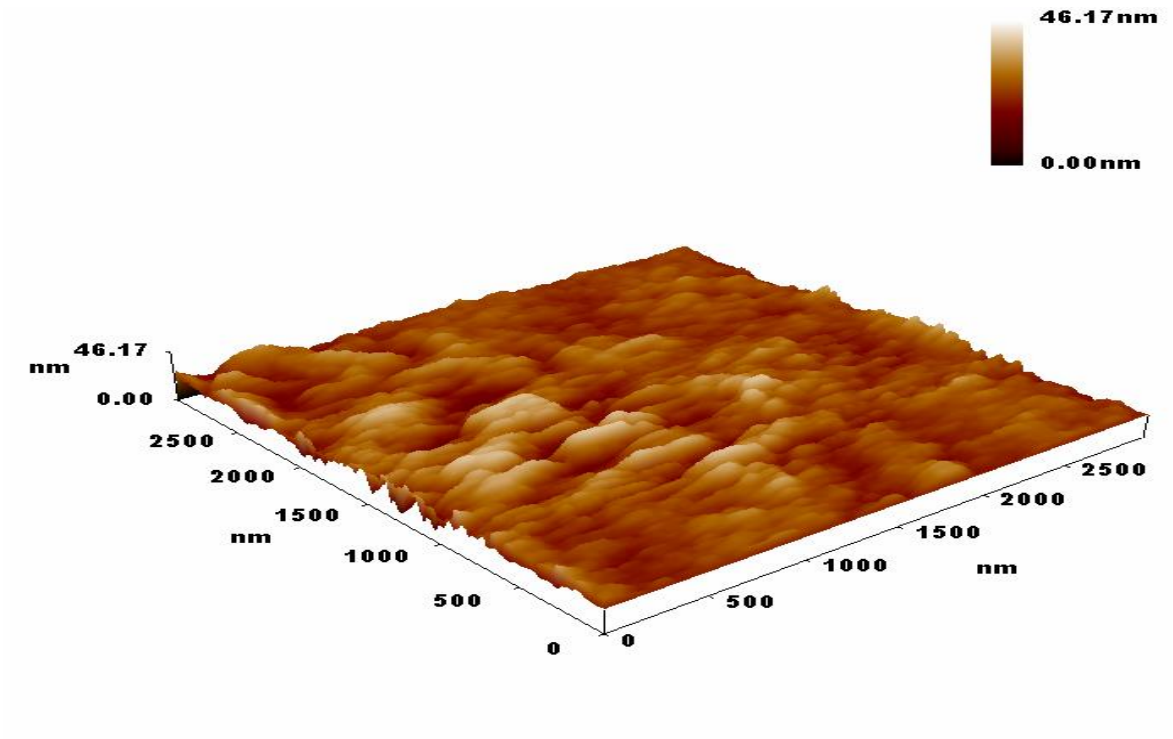

Figure 3: 3D image AFM of biosynthesized AgNPs

UV-Visible Spectrophotometer (UV-Vis)

The UV-vis spectroscopic analysis of biosynthesized AgNPs illustrates in figure **4** revealed a peak with maximum absorbance at 454 nm.

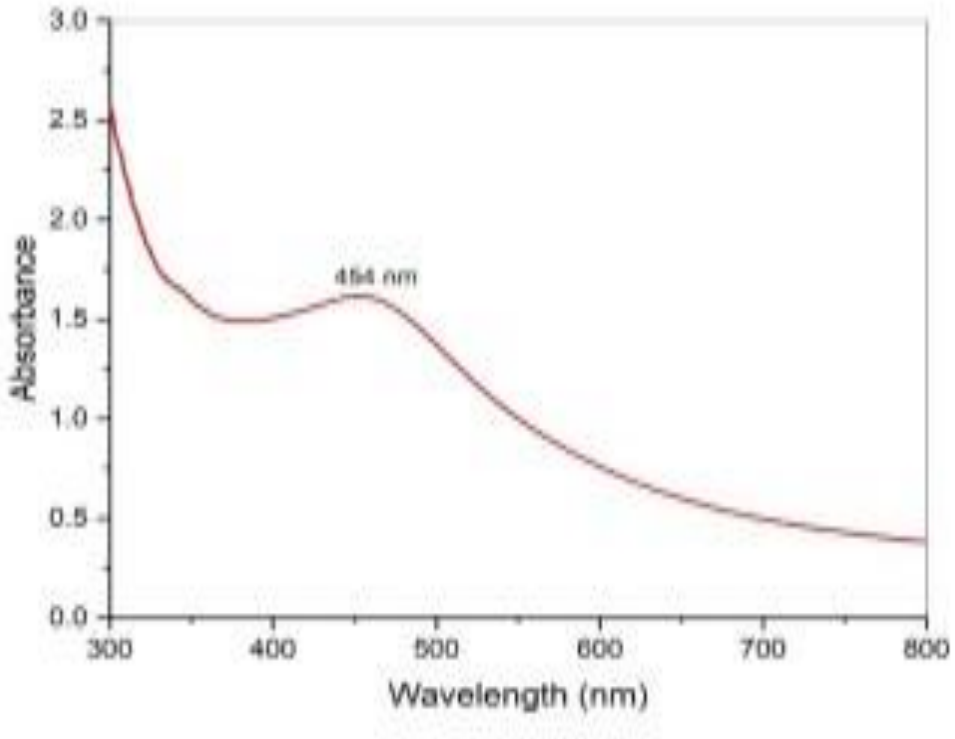

Figure 4: UV−visible absorption spectrum of AgNPs

Fourier Transform IR Analysis (FTIR)

Figure **5** from a fourier transform infrared analysis of biosynthesized AgNPs showed the presence of 15 bands, namely at (3847.15, 3738.61, 3671.03, 3265.99, 29181.15, 2850.37, 2355.65, 1738.32,1645.44, 1536.15,1136.31, 1076.67, 811.42, 668.51, 544.07) cm−1.

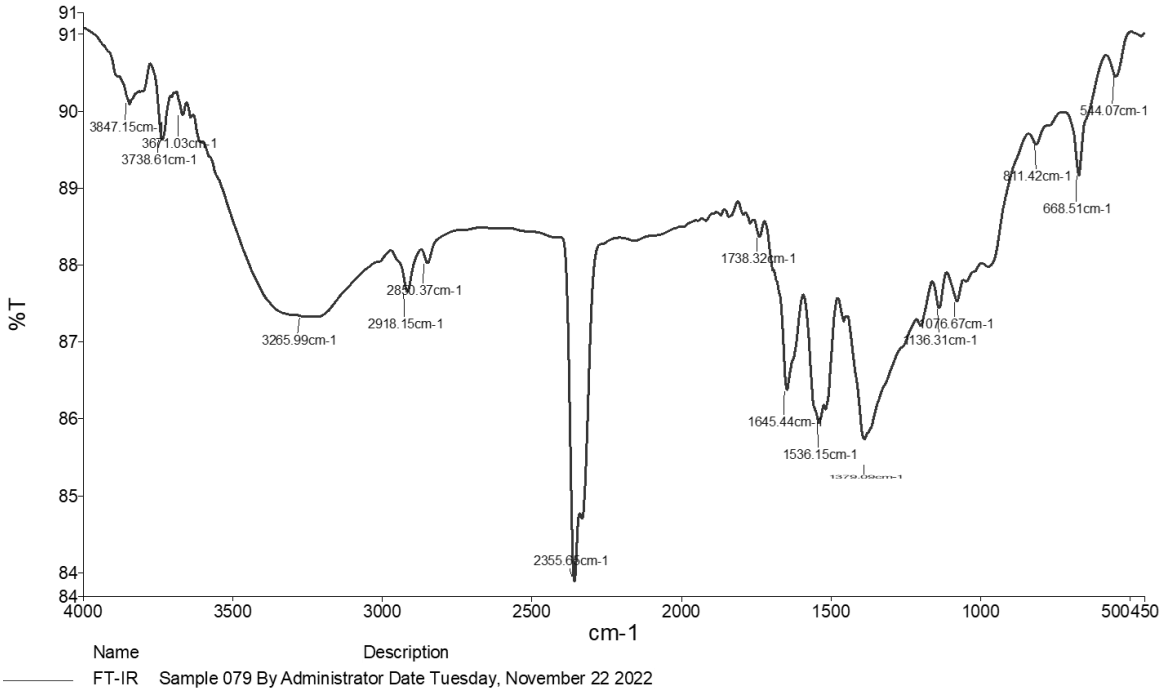

Figure 5: FTIR of AgNPs synthesized by *P. aeruginosa*

Field emission scanning electron microscopy (FE-SEM)

The morphology of NPs is an important aspect that contributes to the physiochemical properties of the substances the FE-SEM analysis was used to observe the physical appearance and the aggregation state of the synthesized AgNPs. Figure **6** represents the FE-SEM image used to investigate the morphological properties of AgNPs, with particle dimensions ranging between (19.00 - 23.72) nm.

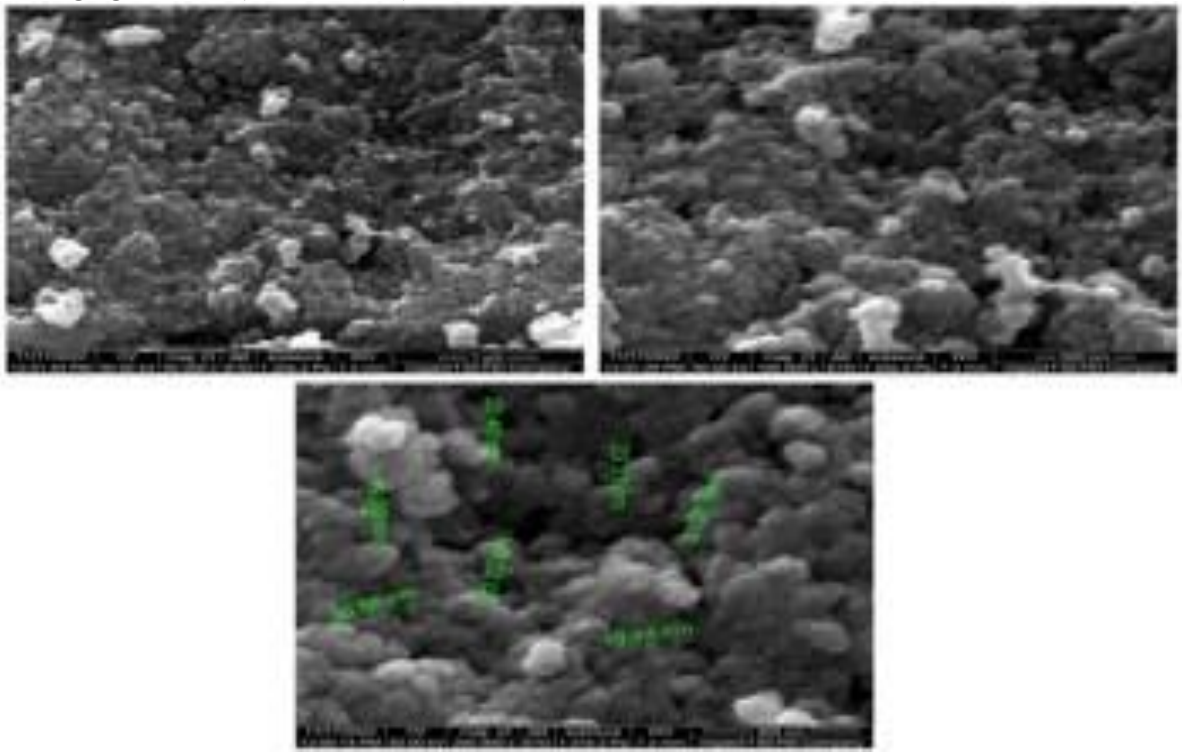

Figure 6: FE-SEM image of silver nanoparticles synthesized by *P. aeruginosa* (50000X) (100000X) (200000X)

X –ray Diffraction (XRD)

Popular analytical method X-ray diffraction (XRD) has been used to examine both molecular and crystal structure, any crystal that receives an X-ray reflection will produce a variety of diffraction patterns as shown in figure **7.**

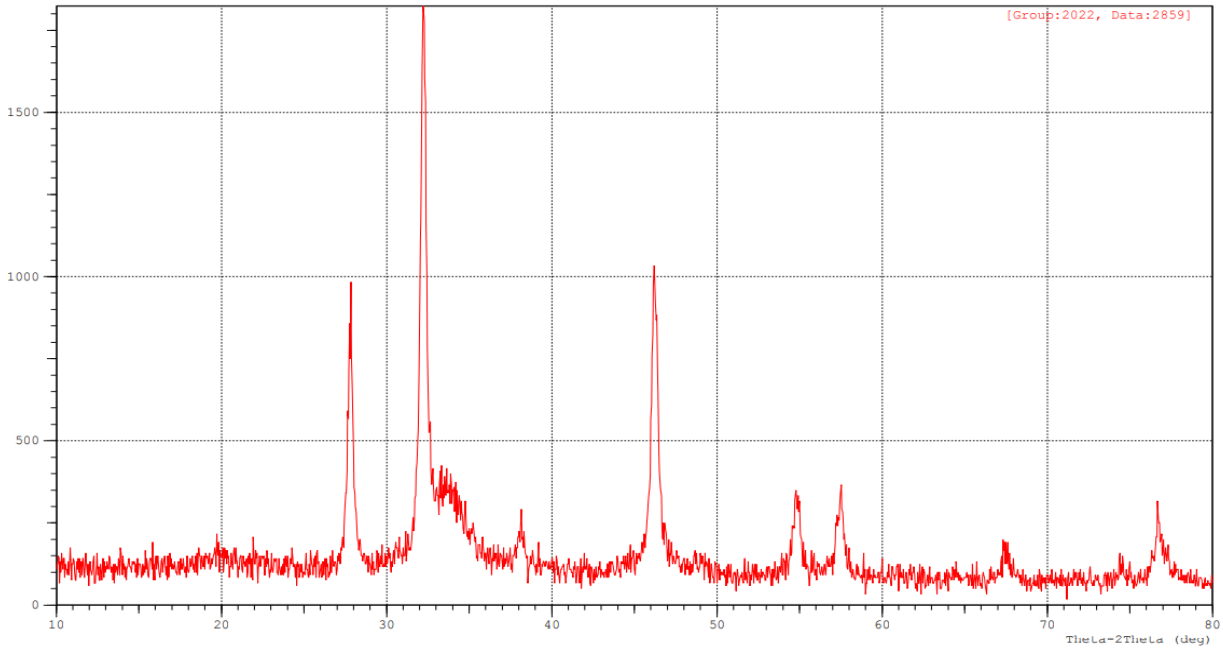

Figure 7: XRD pattern of AgNPs synthesized by *P. aeruginosa*

The antimicrobial activity of melanin and silver nanoparticles against UTI pathogenic microorganisms

The results show different inhibitory zones in reaction to different concentration of AgNPs and the synergistic effects of AgNPs and melanin. The results of silver nanoparticles and combination with melanin against these isolates showing in table **2** and Figure **(8,9,10)**ز

Table 2: The effect of silver nanoparticles and combination it with melanin on UTI pathogenic microorganisms

Type of organism	antimic robe	1024 µg/ml	512 µg/ml	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml
C. albicans	**AgNPs**	22 mm	18 mm	10 mm	8 mm	8 mm	8 mm	4 mm	2 mm
	Mix	32 mm	30 mm	28 mm	20 mm	16 mm	16 mm	12 mm	12 mm
S. haemolyticus	**AgNPs**	22 mm	20 mm	10 mm	8 mm	2 mm	2 mm	2 mm	2 mm
	Mix	28 mm	20 mm	20 mm	14 mm	12 mm	10 mm	10 mm	8 mm
S. aureus	**AgNPs**	18 mm	18 mm	12 mm	10 mm	8 mm	8 mm	6 mm	2 mm
	Mix	20 mm	20 mm	12 mm	12 mm	10 mm	8 mm	8 mm	4 mm
E. faecalis	**AgNPs**	10 mm	10 mm	6 mm	4 mm	4 mm	4 mm	0 mm	0 mm
	Mix	14 mm	12 mm	6 mm	6 mm	6 mm	6 mm	4 mm	2 mm
E. coli	**AgNPs**	18 mm	14 mm	10 mm	8 mm	0 mm	0 mm	0 mm	0 mm
	Mix	24 mm	20 mm	14 mm	12 mm	10 mm	8 mm	8 mm	4 mm
P. mirabilis	**AgNPs**	8 mm	8 mm	4 mm	2 mm	0 mm	0 mm	0 mm	0 mm
	Mix	14 mm	14 mm	10 mm	10 mm	4 mm	4 mm	2 mm	0 mm
A. baumannii	**AgNPs**	12 mm	12 mm	6 mm	4 mm	2 mm	2 mm	2 mm	0 mm
	Mix	18 mm	16 mm	10 mm	10 mm	6 mm	6 mm	4 mm	2 mm

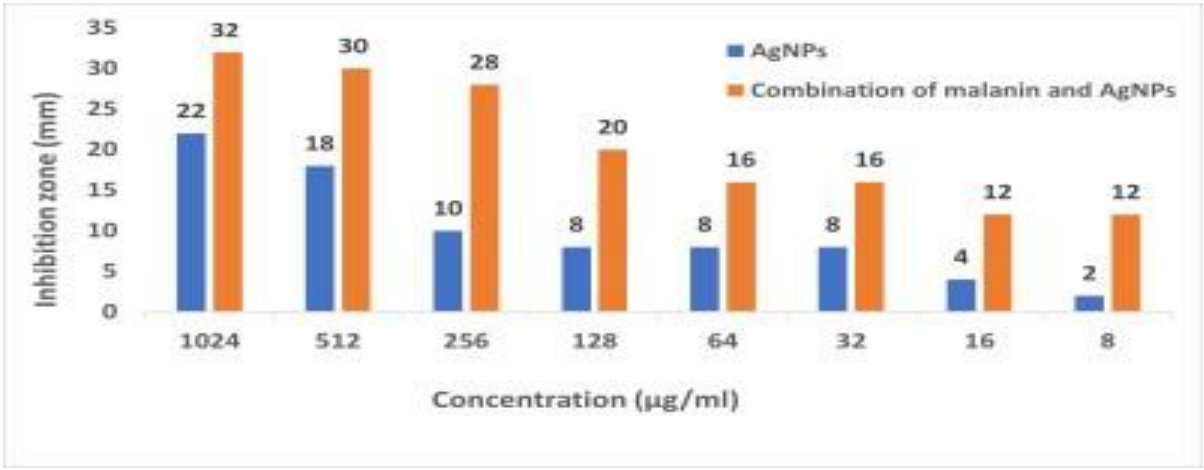

Figure 8: The synergistic effect of AgNPs and melanin on *C. albicans*

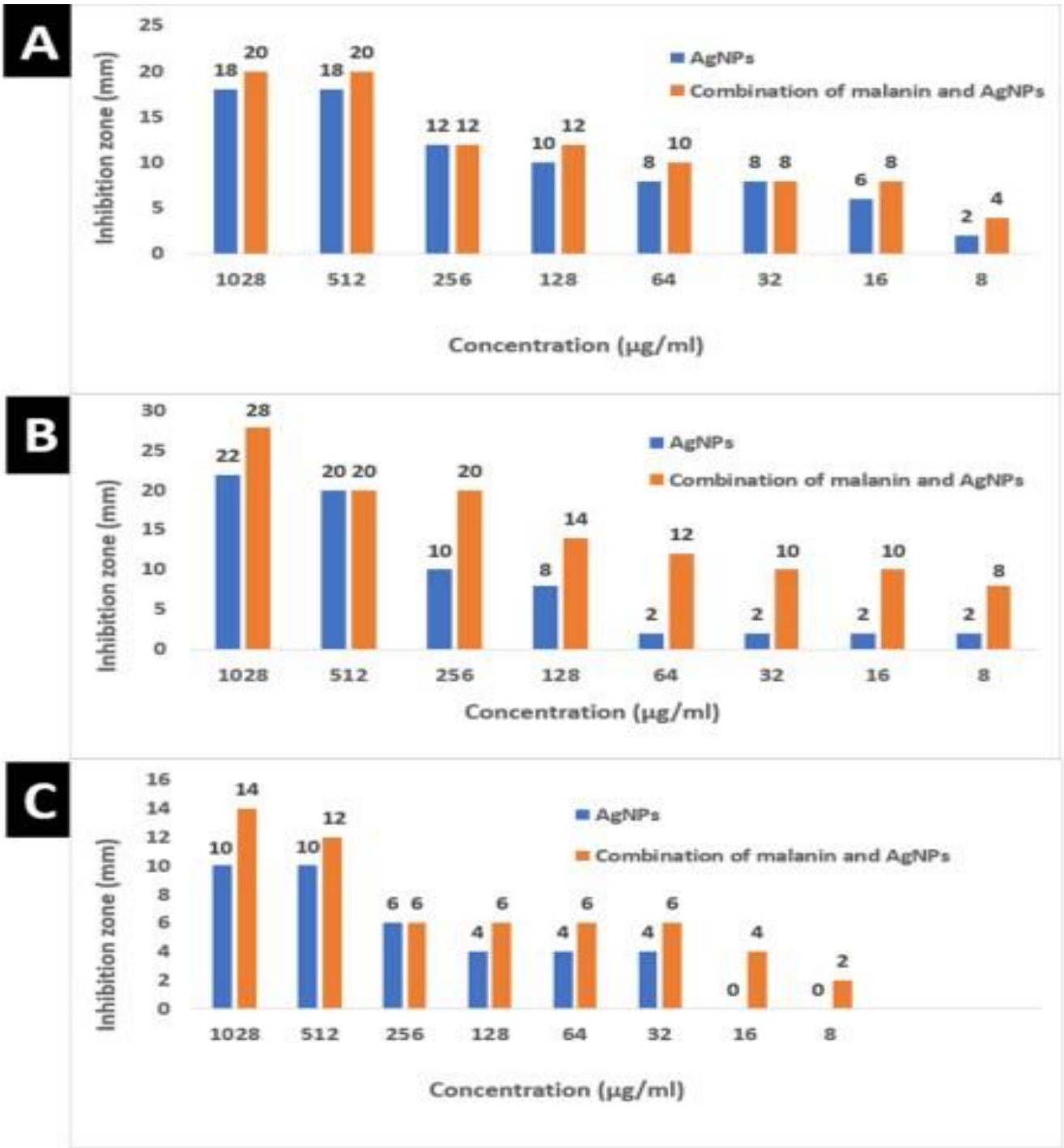

Figure 9: The synergistic effect of AgNPs and melanin on (A) *S. aureus* (B) *S. haemolyticus* (C) *E. faecalis*

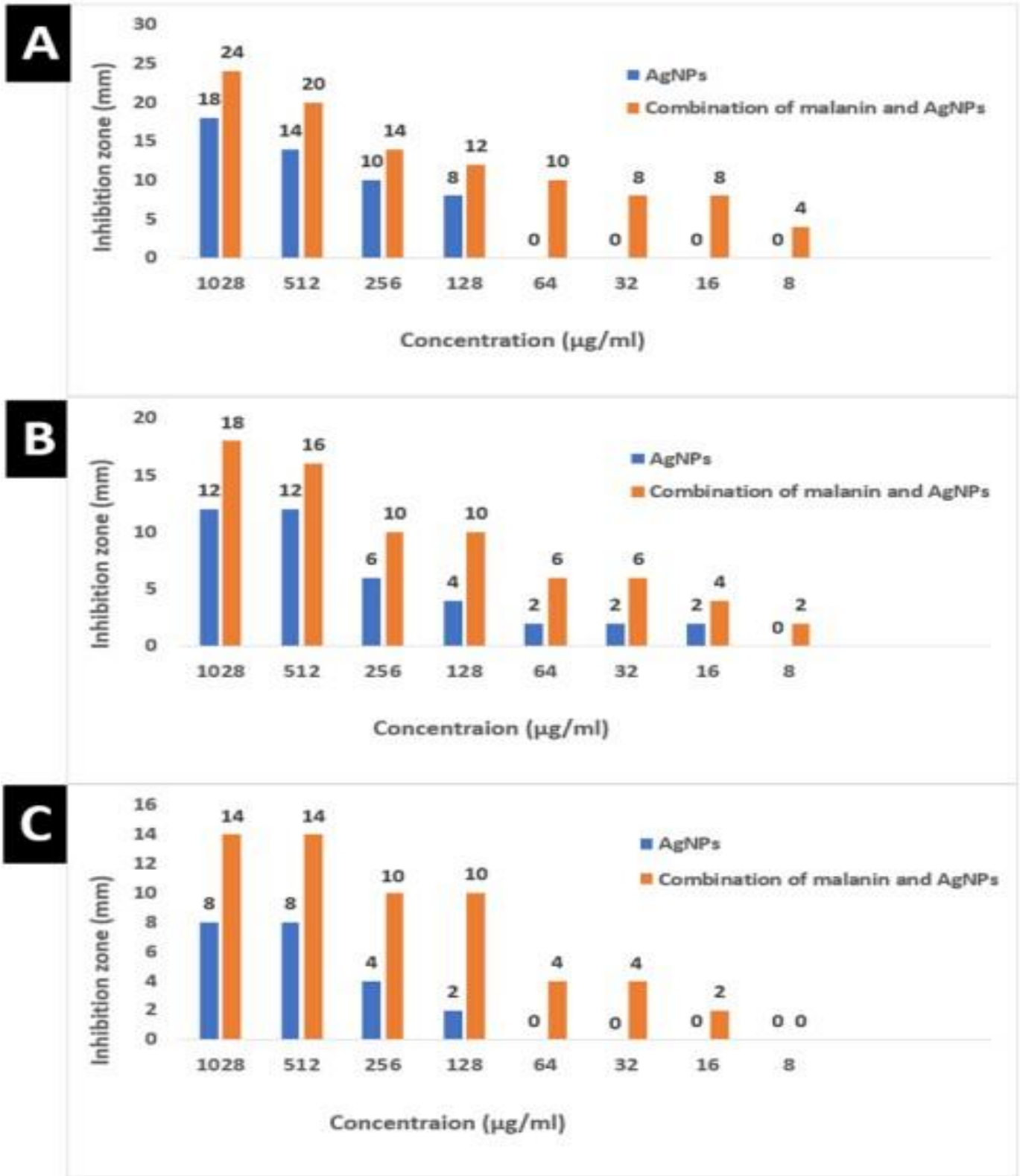

Figure 10: The synergistic effect of AgNPs and melanin on (A) *E. coli* (B) *A. baumannii* (C) *P. mirabilis*

DISCUSSION

Only 35(32.11%) isolates were *P. aeruginosa* were obtained from 109 clinical samples while other 74(67.88%) represented other bacterial genera. These isolates appear as smooth mucoid as mention by **Khalil *et al.*** [17] as pale colony on MacConkey agar and ability to produce pyocyanin pigment similar as mentioned by **Hossain *et al.*** [18]. The biochemical tests are similar to those described previously by **Tawakol *et al.*** [19].

The screening found four isolates that are three from burns and one from patient in ICU with tracheostomy are ability to produce melanin shown in figure **1** as used by **Vijayan *et al.*** [20]. The extraction was done by two basic steps acid precipitation and centrifugation as shown in figure **2**. Silver nanoparticles synthesis from *P. aeruginosa* suspension growth and characterization by atomic force microscopy that showed average diameter (46.17) nm as shown in figure **3**. The average particle diameter according to **Hafez *et al.*** [21] that mention size range between 5 and 45 nm. while average particle size is 68.13 nm according to **Chauhan *et al.*** [22]. Figure **4** revealed a peak with maximum absorbance at 454 nm. A similar study found the highest absorption peak at 429 nm [23].

Figure **5** showed the presence of 15 bands. The band is around 3847.15, 3738.61 cm-1 due to the peptide linkages' N-H stretch vibrations or the hydroxyl group of the carboxylic acid. The peaks 3671.03, 3265.99 cm-1 correspond to O-H stretching vibration, indicating the presence of alcohol and phenol. The peaks at (29181.15, 2850.37) cm-1 can be assigned to the C–H- aromatic compound stretching. The band at 1738.32 cm-1 was assigned for C-C stretching (non-conjugated). Stretching of carbonyl groups (C=O) is attributed to the peak at 1645.44 cm1, whereas bands in spectrum 1536.15, 1076.67 cm-1 were assigned for N-H and C-N (amines) stretch vibration of the proteins, respectively, and at 544.07 cm−1corresponds to C–Cl stretching in the alkyl group these peaks results are similar with **Rajeshkumar *et al.*** [24]. It was noted that the shapes of the samples ranging from spherical to hexagonal and illustrated a lower agglomeration degree as in figure **6**.

This image is similar to **Nalwade *et al.*** [25], who mentioned that the particles range in size from 11.2 to 50.12 nm, while the synthesized silver nanoparticles according to **Singh *et al.*** [26] were spherical and had an average diameter of 27.4 nm. From 20^0 to 80^0, diffracted intensities were measured, and lattice planes were observed of (100), (002), (101), (102), (110), (103) and (112) corresponded to the 2θ values of 27.87°, 32.03°, 46.57°, 54.91°, 57.53°, 67.89°and 77.15° respectively as in figure **7**. These peaks are a result of the organic compounds in the extract that reduce the amount of silver ions and stabilize, the resulting agree with **Singh *et al.*** [26] and as **Aravinthan *et al.*** [27].

The antimicrobial activity of silver nanoparticles as shown in table **2** Figure **(8,9,10)** found that the strongest inhibition zone for all test microorganisms was at a concentration (1024 µg/ml), which was (22) mm for *C. albicans* and *S. haemolyticus* (18) mm for *S. aureus*, and *E. coli* (12) mm for *A. baumannii* and (10) for *E. faecalis* and (8) mm for *P. mirabilis*.

While the weakest inhibition zone was at a concentration (8 µg/ml) with a diameter of (2) mm for *S. aureus, S. haemolyticus*, and *C. albicans.* And (4) mm at a concentration (32 µg/ml) mm for *E. faecalis* and (8,2) mm at a concentration (128 µg/ml) for *E. coli* and *P. mirabilis* respectively. And (2) mm at a concentration (16 µg/ml) for *A. baumannii*. The largest inhibition zone was detected against *E.coli* (20.5mm) while the smallest was 9.5mm was against *Staphylococcus aureus* [28]. Smaller dimensions of AgNPs (<30 nm) were found to be optimal against *S. aureus*, as mentioned by [4]. AgNPs exhibited antibacterial activity against *A. baumannii* as mentioned by [26].

There are several mechanism of silver nanoparticles according to **Tawfeeq *et al.*** [29] the first mechanisms is AgNPs interactions with the thiol group of L-cysteine protein residues will lead to enzymatic dysfunction. The second, positive 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. Finally, the silver nanoparticles cause damage on proteins and DNA via release of reactive oxygen species (ROS)

The synergistic effects of AgNPs and melanin showed the highest antimicrobial activity in all isolates in comparison with sliver nanoparticles alone as shown in table **2**. According to figure **8** combination of melanin and AgNPs against *C. albicans* showed a maximum inhibition zone with a diameter of (32 mm) at a concentration 1024 µg/ml in comparison with inhibition zone diameters (12 mm, 22 mm) for melanin and AgNPs alone respectively. In contrast, the minimum inhibition zone was at a concentration 8 µg/ml with a diameter of (12 mm).

Combination of melanin and AgNPs against Gram-positive bacteria as in the figure **9** showed a maximum inhibition zone at concentration 1024 µg/ml with a diameter of 28 mm, 20 mm and 18 mm respectively. A concentration (8 µg/ml) gives the minimum inhibition zone with a diameter of 4 mm, 8 mm, and 2 mm for each isolate respectively.

For Gram-negative bacteria as in figure **10** showed a maximum inhibition zone was also at a concentration 1024 µg/ml with a diameter of (24 mm, 18 mm, and 14 mm) for each isolate respectively. While minimum inhibition zone was at a concentration 8 µg/ml with diameter equal to (2 mm) for *E. coli* and (4 mm) for *A. baumanni*. While *P. mirabilis* showed the minimum inhibition zone (2 mm) at a concentration 16 µg/ml. The melanin-mediated silver nanoparticles displayed antibacterial as well as antifungal activities,

as mentioned by **Patil *et al.*[30]**. A maximum zone of inhibition was recorded for *S. aureus* (22mm), as mentioned by **Gurme *et al.*[31]**. As mentioned by **Macieja *et al.*[32]** melanin act as a metal salt reducer and further stabilizes the nanoparticles (acting as a capping agent), and their antimicrobial activity is largely due to the induction of pores in cell membranes, which is attributed to the interaction of silver with sulfur-containing membrane proteins.

CONCLUSIONS

Biosynthesized silver nanoparticles (AgNPs) by *P. aeruginosa* has antimicrobial activity against UTI pathogenic microorganisms. There is a positive correlation between silver nanoparticles and melanin as synergistic activity against UTI pathogenic microorganisms because melanin act as a reducing/capping agent for silver nanoparticles as results increase the antimicrobial activity of silver nanoparticles.

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