

RESEARCH PAPER

Characterization and Antimicrobial Activity of Walnut Shell Nanoparticles

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ABSTRACT

With an increasing of Antibacterial problems for aging population and resistance, there is more needing for more effective treatments to enhance the quality of denture base. Natural powders have biocompatibility characteristics and considerable mechanical properties as well as being inexpensive and eco-friendly. In this study, walnut shells, an abundant agricultural by-product, were processed into nano-scale powders using high-energy ball milling. The physicochemical properties of the nano-engineered walnut shell powder were characterized by) AFM), X-ray diffraction (EDX), and Fourier transform infrared spectroscopy (FTIR). The agar diffusion method was used to evaluate the antibacterial activity against selected Gram-positive and Gram-negative bacteria and pathogens. minimum inhibitory concentration (MIC) assay was done to evaluate the antibacterial activity and the result was showed that the walnut shell nanoparticles demonstrated powerful antibacterial properties, with zones of inhibition growing proportionally with concentration. The enhancement of bioactivity which represented as increasing in penetration of cell membranes at the nanoscale and increasing in surface area, beside that increasing in active phenolic content were observed. According to these results, walnut shell nanoparticles could be used in many applications as food preservatives, biomedical materials, and environmental disinfection which is consider ecofriendly, and natural antibacterial agent.

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INTRODUCTION

Recently Nanomaterials especially plant waste nanoparticles are used as antimicrobial by creating reactive oxygen species (ROS), disrupting the cell membrane, and releasing metal ions, all of which damage and kill microbe. These materials can act as standalone agents or as part of composite therapies, enhancing the effectiveness of traditional antibiotics and helping to overcome antibiotic resistance, Particularly, walnut shell nanoparticles

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which shown exceptional antibacterial efficacy against a variety of harmful fungus and bacteria [1]. Because the increasing of bacterial resistance to antibiotics and raising the side effects of these antimicrobe and due make a sustainable and environmentally friendly alternatives of synthetic preservatives. Walnut shells are one such natural material; they are rich in bioactive compounds such as lignin, tannins, and polyphenols. Walnut shell nanoparticles (WSNPs) are interesting



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candidates for antibacterial applications due to their increased surface area and reactivity at the nanoscale [2].

Last ten years walnut shells were one on the important natural compound that received a great attention due to their enormous applications in biological, pharmaceutical, environmental and biomedical fields. Among these applications, the antibacterial potential of walnut shell nano powders has received particular attention [3] Walnut shell powders have a larger surface area and developed reactivity, enhancing their ability to inhibit bacterial growth, when it processed at the nanoscale. The results of the previous studies have shown that walnut shell nano powders effectively decrease the growth of common pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus*, making that natural products as a promising and environmentally friendly antibacterial material [4].

The studies in last couple years showed that walnut shell-derived nanoparticles, especially those containing hydrocar, silver, and copper, exhibit exceptional antimicrobial activity against a variety of pathogens, such as *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The promising potential of walnut shell-derived nanoparticles as effective, biodegradable, and low-cost antimicrobial agents and ecofriendly encourages the development of novel approaches to antimicrobial control in the food, pharmaceutical, and agricultural sectors [5,6]. Nanoparticles have demonstrated encouraging potential in the fields of agriculture (e.g., nano-fertilizers and pest control), medicine (e.g., antibacterial and anticancer), and environmental remediation (e.g., removal of heavy metals and dyes from wastewater) [7].

The phenolic compounds derived from walnut shells have powerful antibacterial properties against *Staphylococcus aureus* and *E. coli*, indicating possible uses in natural antibacterial formulations, particularly in food packaging [3]. According to Wang et al. (2019) and Zhang et al. (2021) which revealed that decreasing walnut shell particles to the nanoscale markedly improved and enhance their antibacterial activity, predominantly by disrupting bacterial cell walls [4]. Also the high surface area and abundance of bioactive chemicals in walnut shell biochar and Nano powders gave them remarkable antibacterial qualities, indicating their applicability for antimicrobial coating and water purification applications [8]. A new approach for biomedical application was studied also in large scale and with many nanoparticles especially mineral oxide like zinc oxide silver oxide and others like Lee and Kim (2018), who examined the synergistic activity of walnut shell powder and silver nanoparticles. When their results crowned that the two substances significantly enhanced antibacterial activity against strains of bacteria that are resistant to many drugs [9]. This study investigates the development, characterization, and antibacterial activity of walnut shell nanoparticles to highlight their value as a green alternative in the fight against microbial illnesses.

MATERIALS AND METHODS

Materials used

Walnut shells were used as the primary source material in this research after processing as a nanomaterial. The procedure of manufacturing walnut shell nanoparticles begins with crushing walnut shells, then the material was softened and the particle size was reduced using a mortar and pestle. At the end of the process the nanoparticles



Fig. 1. Walnut shells before and after grinding process.

were extracted using an electric grinder. In order to have an appropriate size for nanoparticles, the electric grinder and mortar were used.

Preparation Method (Work Procedure)

After collecting the walnut shells, they washed with water to remove dirt and any particle that not desired, and air-dried at room temperature for 24 hours until completely dry. By using the conventional hand grinder, the dried shells were initially ground obtain a coarse powder, which was further reduced in size and softened using a mortar and pestle. The pre-ground material was then processed using a high-speed electric grinder for 4–6 hours to achieve nanoscale particles. The resulting powder was sieved through a fine-mesh sieve to separate nanoparticles from larger particles, ensuring sample uniformity. The final walnut shell nanoparticles were stored in clean, dry, airtight glass containers until required for experimental use.

Antibacterial activity

Bacterial strains

The nano walnut shells antibacterial activity was tested by using bacterial strains provided from the culture collection of Biotechnology Dept. Baghdad University, Baghdad, Iraq. two pathogenic strains of Gram positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and two strains of Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria.

Inoculum preparation

Each bacterial strain growth harvested from subculture overnight at 37 °C in Mueller-Hinton agar slants by using 5 ml of sterile saline water, its absorbance was adjusted at 580 nm and diluted to attain viable cell count of 10⁷ CFU/ml using spectrophotometer.

Antibacterial activity of nano walnut shells

Agar well diffusion assay was performed to investigate antibacterial efficacy of nano walnut shells against two species from gram positive and two species from gram negative bacteria. 24 hours old Nutrient broth cultures of test bacteria were swabbed uniformly on sterile Nutrient agar plates. Using sterile corn borer, wells of 5mm diameter were punched in the inoculated plates. 50µl of nano walnut shells (1mg/ml of deionized distilled water, sterilized through Millipore filter (0.22 µm were

added, labeled wells and the plates were incubated for 24 hours at 37°C. The zones of inhibition around the wells were measured using a ruler [10].

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MICs) of nano walnut shells was measured using a Resazurin -based turbidimetric (TB) assay (8) The products were serially diluted twofold from (1mg/ml to 0.97 µg/ml for nano walnut shells. All sterility control wells for all tested bacteria remained blue after an overnight incubation followed by a 2-hour incubation with resazurin. In comparison, all wells in the growth control column (which included both growing bacteria and medium) of all tested microorganisms had transformed from blue to pink or pale pink. The MIC defined as: The lowest concentration of nano walnut shells which inhibited the visible growth after incubation for 18 hrs. at 37 °C.

RESULTS AND DISCUSSION

Atomic Force Microscopy (AFM) Analysis of Walnut Shell Nanoparticles

The atomic force microscopy (AFM) analysis was made to evaluate the shape characteristic and size distribution of walnut shell nanoparticles. The two-dimensional particle analysis map and three-dimensional surface topology, particle size histogram, and statistical data were demonstrated in Fig. 1 which shows that the average diameter of the walnut shell nanoparticles was 19.29 nm, with particle sizes ranging from 2.418 nm to 147.2 nm. A total of 658 particles were identified within the scanned region of 1664 nm × 1664 nm, related to a particle density of 23,757,130 particles/m². The surface coverage was approximately 16.5%, indicating moderate particle dispersion on the analyzed surface.

The particle size distribution that falls within smaller diameter range (<40 nm), leading to narrow distribution with a bias toward smaller particles this result appears in the histogram (bottom right of Fig. 2) in which the classification of projected area detected most of the particles as small, with only a few falling into the medium or large categories. The shape and surface roughness of the nanoparticles, reveals a maximum particle height (Z-max) of 395.4 nm and an average Z-height of 228.7 nm and this result was confirmed with 3D topographic image (top right).

EDX Analysis of Walnut Shell-Based Nanoparticles

Oxygen (O) was identified as the predominant element in the walnut shell powder, showing normal weight percentage of 79.23% with atomic percentage of 85.45%. this high oxygen content is expected because of the organic nature of walnut shells, which are rich in cellulose, hemicellulose, and lignin. as shown in table 1 which represent the EDX analysis While the Indium (In) appears as the second most prevalent element, which leading to successful incorporation of metal components into the nanoparticle structure, possibly for functionalization or enhancement purposes.

Carbon (C) appears in low weight percentage (1.67%) and atomic percentage (0.19%), which may be attributed to the limitations of EDX in detecting light elements, especially when overlapped with high oxygen content. Antimony (Sb) and aluminum (Al) were detected in trace quantities, which may indicate slight contamination during processing or possible intentional doping. Their presence could affect the surface characteristics or electronic properties of the nano powder.

Further insights into the elemental composition and unique distribution of elements in the walnut

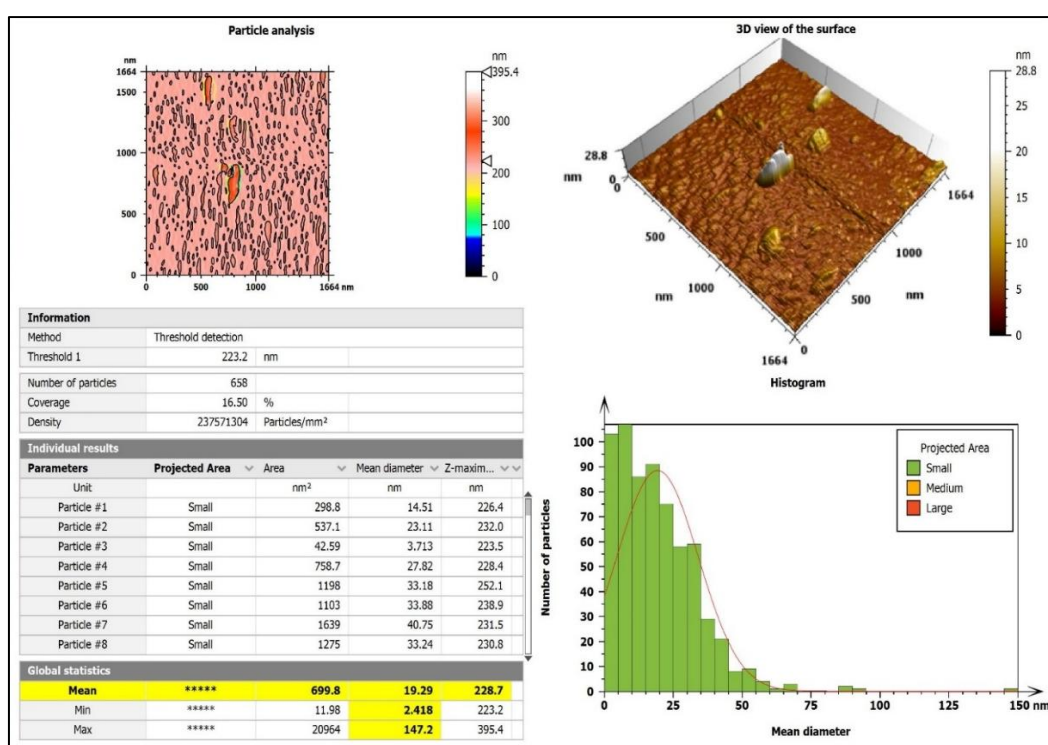


Fig. 2. AFM analysis of walnut shell (W.S.) nanoparticles.

Table 1. EDX-based chemical composition of walnut shell powder.

Element	Element Name	Series	C norm. (wt.%)	C Atom (at. %)	C Error (wt.%)
O	Oxygen	K-series	79.23	85.45	11.25
In	Indium	K-series	17.25	13.97	4.59
C	Carbon	L-series	1.67	0.19	0.12
Sb	Antimony	L-series	1.32	0.14	0.11
Al	Aluminum	K-series	0.52	0.25	0.08

shell nanoparticle sample provided in Fig. 3.

At the (top) in EDX spectrum strong signals for carbon (C) and oxygen (O) in the low-energy region (0.2–0.5 keV) were shown, supporting the organic nature of the walnut shell matrix. Additionally,

successful incorporation or surface decoration of the metal nanoparticles is due to the presence of peaks that corresponding to indium (In) and antimony (Sb) at higher energies (~3.1–4.0 keV).

The spatial distribution of each element

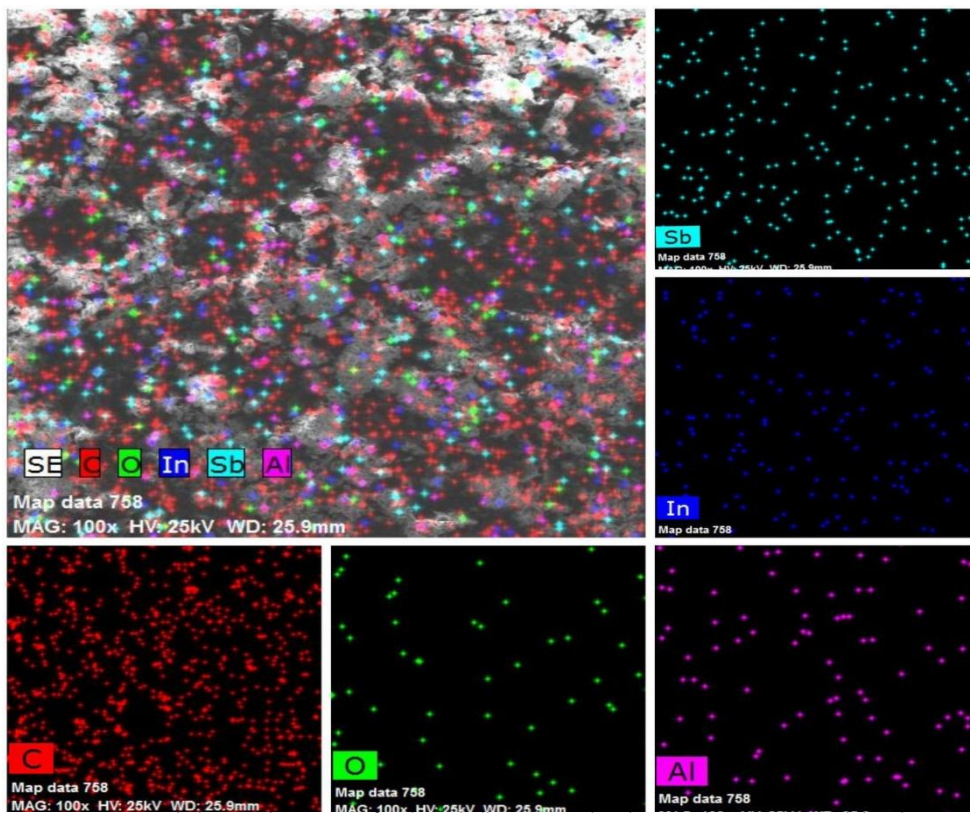
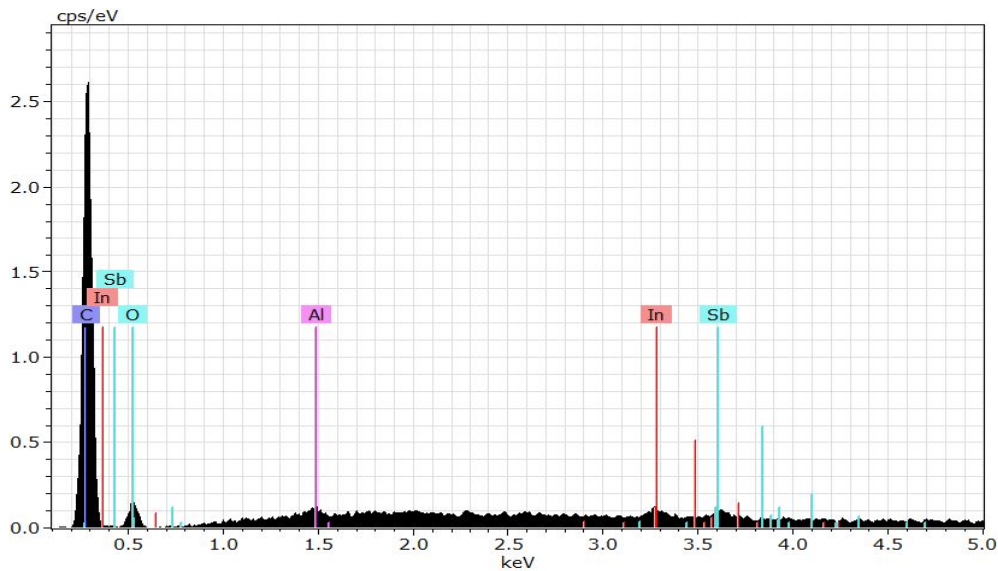


Fig. 3. EDX spectrum and elemental mapping of walnut shell nanoparticles showing the presence and distribution of C, O, Al, In, and Sb.

was shown in the element mapping images (bottom). Carbon and oxygen appear evenly distributed across the surface, consistent with a biomass-derived material. Aluminum is distributed in isolated patches, while the Indium and antimony found more dispersed and patchy distributions, indicating the formation of fine metal nanoparticles. These results support the conclusion that the sample is a hybrid organic-inorganic nanocomposite, with mineral elements finely incorporated or deposited on the natural walnut shell matrix.

FTIR Analysis of Walnut Shell Nanoparticles

The walnut shell nanoparticles have many effective functional groups that derived from plant biomass like lignin, cellulose, and hemicellulose as shown in absorption bands of FTIR spectrum Fig. 4. This broadening in the 3400 cm^{-1} range is lead to the stretching vibrations of hydroxyl groups (O–H), and this functional group are very common in cellulose and lignin structures because of the presence of alcohols and phenols. This indicates the strength of hydrogen bonds within the structure, while the peak at 2920 cm^{-1} is related to the C–H and that CH_2 aliphatic stretching bands are common in cellulose and

hemicellulose. Peak at 1730 cm^{-1} : This absorption is associated with the C=O stretching frequencies of ester or carboxylic acid groups, which are often found in hemicellulose, or due to the oxidation of lignin components. The lignin content of the walnut shell material representing with aromatic C=C stretching found at the sharp band of 1600 cm^{-1} : and another aromatic structure generated by lignin appears at the peak approximately 1510 cm^{-1} : while aryl ether or esters shown at the band 1240 cm^{-1} : which associated with C–O stretching vibrations. A peak near 1030 cm^{-1} : Indicates the stretching of cellulose and hemicellulose across C–O–C bonds, a characteristic of polysaccharide structures. At last, β -glycosidic bonds between glucose units in cellulose represented in small peak near 890 cm^{-1} .

Antibacterial Effect and determination of Determination of minimum inhibitory concentration (MIC)

The antibacterial screening of the walnut shell nanoparticles against four bacterial strains (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*) revealed species-dependent sensitivity. *Staphylococcus aureus* showed

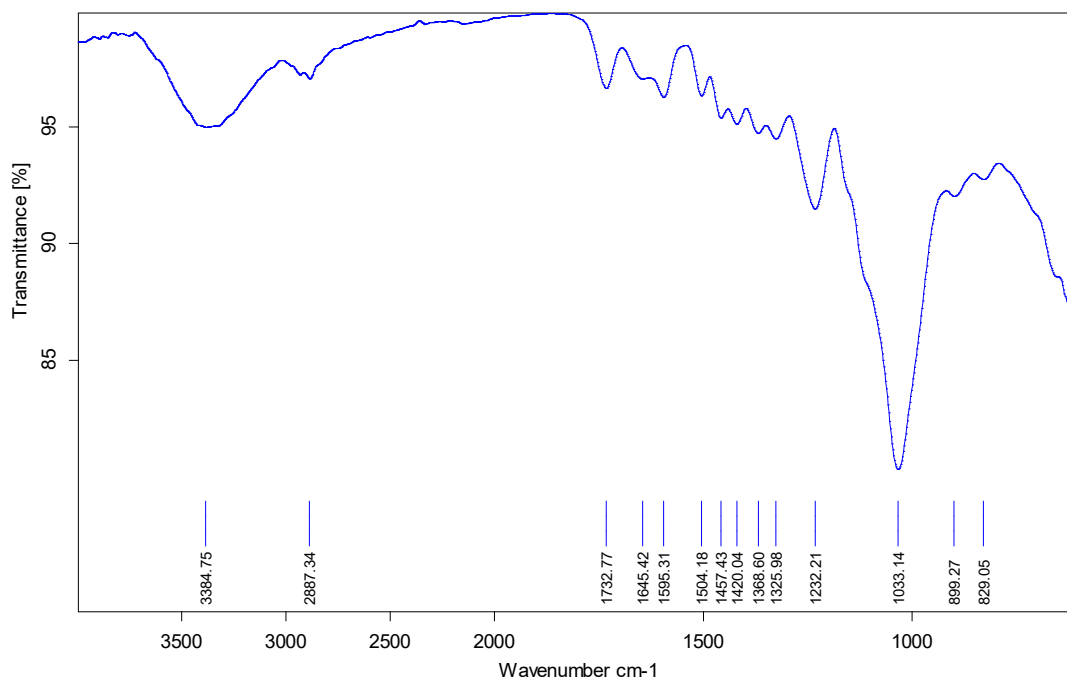


Fig. 4. the infrared spectrum of the walnut shell nanoparticles exhibits distinct functional groups such as hydroxyl, carbonyl, and aromatic, confirming their lignocellulosic nature.

the largest inhibition zone, indicating strong susceptibility, *Enterococcus faecalis* exhibited moderate inhibition, reflecting its known intrinsic resistance, while *Escherichia coli* was moderately inhibited as shown in Fig. 5 suggesting partial sensitivity. Finally, *Pseudomonas aeruginosa* showed little to no inhibition, confirming its high resistance. Overall, the tested compound was more effective against Gram-positive bacteria, especially *S. aureus*, while Gram-negative bacteria showed variable responses, with *P. aeruginosa* being the most resistant, and this may be due to the complexity composition of Gram-negative bacteria and bacterial cell wall structure which led to restricts penetration of many antimicrobial agents. *P. aeruginosa*, in particular, is well known

for its multidrug resistance due to efflux pumps, biofilm formation, and enzymatic inactivation mechanisms [11,12].

These findings are consistent with previous research indicating that natural extracts or nanoparticle-based antimicrobials often show higher activity against *S. aureus* and *E. coli* but limited activity against *P. aeruginosa*.

The minimum inhibitory concentration (MIC) for each pathogenic bacteria was determined as shown in Fig. 6, showing the effect of different concentrations of walnut shell nanoparticles compound against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*.

This difference in susceptibility is consistent

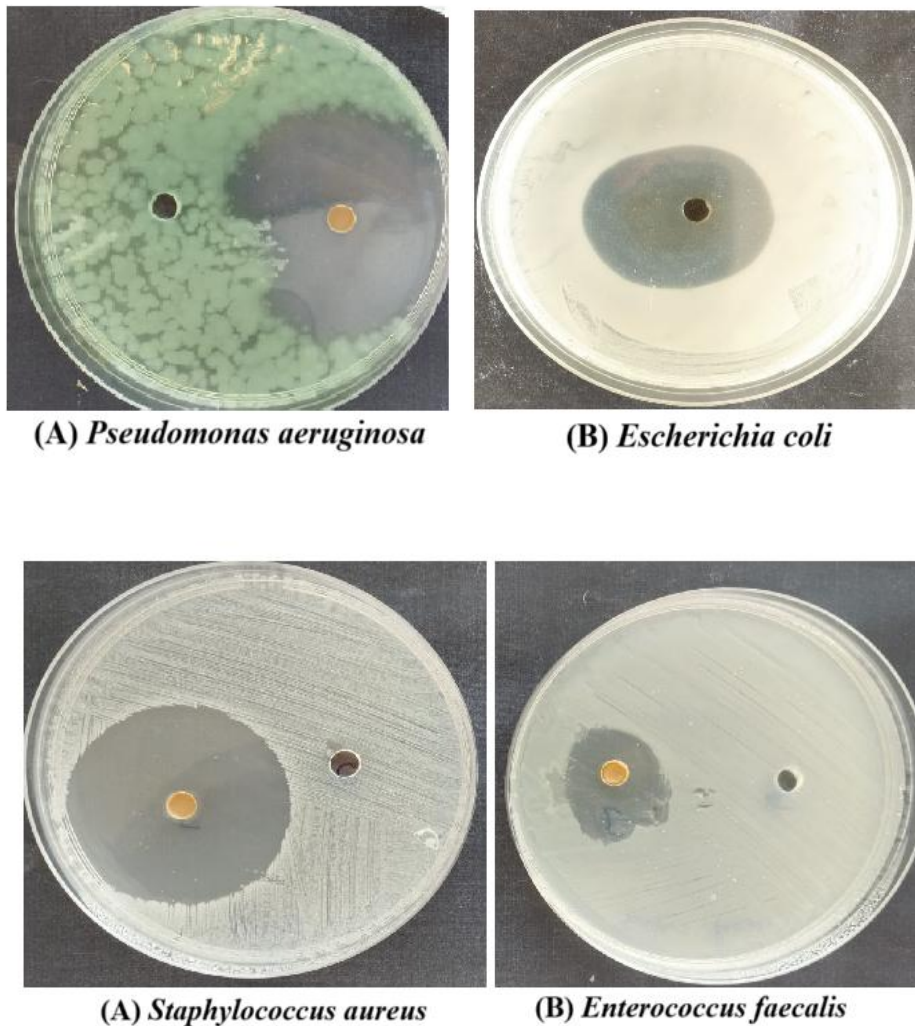


Fig. 5. Comparative Antibacterial Activity of the walnut shell nanoparticles Against Gram-Positive and Gram-Negative Bacteria.

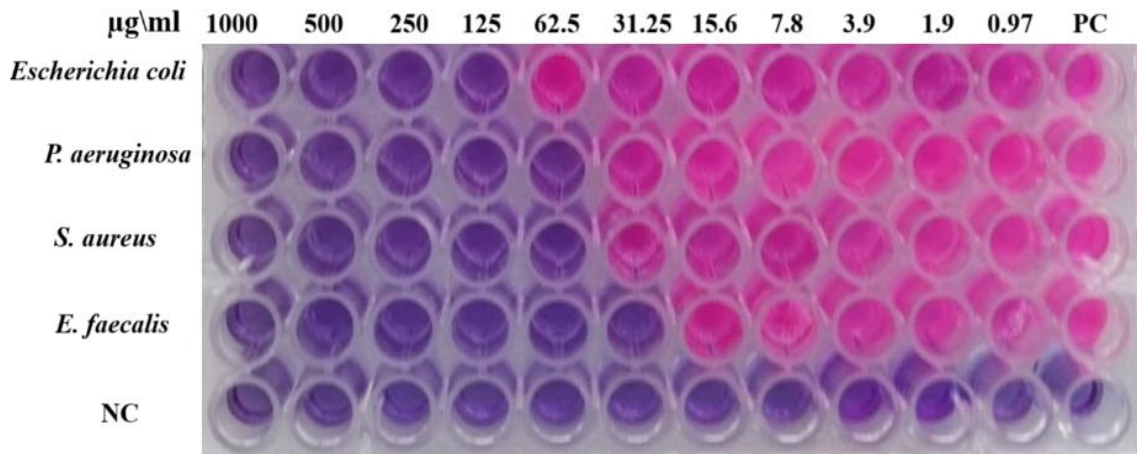


Fig. 6. Minimum Inhibitory Concentration (MIC) Assay of the walnut shell nanoparticles Against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*.

with structural and physiological differences between bacteria gram-positive (*S. aureus*, *E. faecalis*): Susceptibility is generally higher due to the absence of an outer membrane barrier, gram-negative (*E. coli*, *P. aeruginosa*) resistance is stronger, particularly in *P. aeruginosa*, due to efflux pumps, biofilm formation, and impermeable outer membrane [13].

Overall, the tested material demonstrates broad-spectrum antibacterial activity, but with higher efficacy against *S. aureus* and moderate activity against *E. coli* and *E. faecalis*, while *P. aeruginosa* remains highly resistant.

CONCLUSION

In this study, walnut shell nanoparticles (WSNPs) were successfully prepared from natural plant waste using a mechanical grinding method, demonstrating a simple, low-cost, and environmentally friendly approach for producing bio-based nanomaterials. Characterization techniques confirmed the nanoscale nature and chemical composition of the produced particles. AFM analysis revealed that the nanoparticles had an average diameter of approximately 19.29 nm with a size distribution mainly below 40 nm, indicating a high surface area that can enhance biological interactions. EDX analysis confirmed that the material was primarily composed of oxygen and carbon, consistent with the lignocellulosic nature of walnut shells, while trace elements such as indium, antimony, and aluminum were also detected. FTIR analysis further verified the presence of important functional groups such

as hydroxyl, carbonyl, and aromatic structures derived from cellulose, hemicellulose, and lignin, which may contribute to the biological activity of the nanoparticles. The antibacterial assays demonstrated that walnut shell nanoparticles possess noticeable antimicrobial activity against several pathogenic bacteria. Among the tested strains, *Staphylococcus aureus* showed the highest susceptibility, followed by *Enterococcus faecalis* and *Escherichia coli*, whereas *Pseudomonas aeruginosa* exhibited the highest resistance. The results of the agar well diffusion and MIC assays indicate that the antibacterial efficiency of the nanoparticles is influenced by the structural differences between Gram-positive and Gram-negative bacteria, where the outer membrane of Gram-negative bacteria acts as an additional barrier to antimicrobial agents. Overall, the findings highlight the potential of walnut shell nanoparticles as a sustainable and biodegradable antimicrobial material derived from agricultural waste. Their significant activity against important pathogenic bacteria suggests possible applications in biomedical materials, antimicrobial coatings, food preservation, and environmental sanitation. Further studies are recommended to optimize the synthesis conditions, investigate the mechanism of antibacterial action, and evaluate their safety and effectiveness in practical applications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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