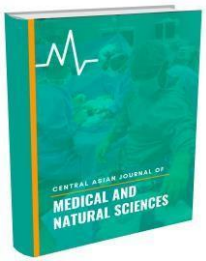

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Article

Enhanced Antibacterial Activity of Artemisia vulgaris Ethanol Extract Using Chitosan-Coated Iron Oxide Nanoparticles Against *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Abstract: Medicinal plants, such as Artemisia vulgaris, have gained increasing attention in traditional and complementary medicine due to their rich content of bioactive phytochemicals with multi-target antioxidant and antimicrobial properties. In recent years, nanotechnology, particularly iron oxide nanoparticles (Fe_3O_4), has emerged as a promising tool to combat multidrug-resistant bacteria. Coating these nanoparticles with chitosan enhances their biostability and antimicrobial efficacy. This study aimed to evaluate the antibacterial activity of ethanol extract of A. vulgaris, Fe_3O_4 nanoparticles, and chitosan-coated Fe_3O_4 nanoparticles against Klebsiella pneumoniae and Staphylococcus aureus. The plant extract was prepared using a Soxhlet apparatus, nanoparticles were synthesized via the co-precipitation method, and chitosan coating was applied at various concentrations. Antibacterial activity was assessed using the disc diffusion method, while XRD, SEM, TEM, and Zeta analysis were employed for characterization. Results showed that the ethanol extract exhibited notable antibacterial activity, with inhibition zones ranging from 18–26 mm. Fe_3O_4 nanoparticles further improved inhibition, and chitosan coating led to a significant increase, reaching up to 35 mm for K. pneumoniae and 34 mm for S. aureus at the highest concentration. Microscopic analyses indicated that chitosan coating enhanced nanoparticle dispersion and prevented aggregation. These findings suggest that combining medicinal plant extracts with chitosan-coated iron oxide nanoparticles offers a promising approach for combating antibiotic-resistant bacteria, opening the way for potential applications in antimicrobial therapy.

Keywords: *Artemisia vulgaris*, Iron oxide Nanoparticles , Ethanol Extract *K.pneumoneiae , S.aureus*

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1. Introduction

Medicinal plants are among the oldest sources of therapy utilized by different civilizations, and today they represent a global focus within the framework of traditional and integrative medicine. The World Health Organization emphasizes that the integration of medicinal plants and herbal preparations into health systems requires evidence-based national strategies, along with biodiversity conservation and assurance of quality and safety [1]. In this context, the Global Traditional Medicine Strategy 2025–2034 was launched to harmonize regulatory policies and strengthen research [1,2].

The therapeutic potential of medicinal plants is attributed to their complex matrix of phytochemical compounds that target multiple cellular pathways. Currently, the network pharmacology approach is widely applied to elucidate the synergistic interactions among these compounds and to better understand the mechanisms of multi-target efficacy and toxicity, supported by artificial intelligence and modern omics data [3,4]. This

methodology is considered a crucial step prior to preclinical and clinical investigations. From a technological perspective, recent years have witnessed remarkable advances in plant extraction methods. In addition to traditional techniques such as maceration and percolation, "green extraction" technologies have become increasingly common, including supercritical CO_2 extraction, ultrasound-assisted extraction, and microwave-assisted extraction, due to their higher efficiency, reduced processing time, and preservation of thermolabile compounds [5,6]. These methods also provide broader applicability for industrial-scale production and sustainable development.

Artemisia vulgaris, belonging to the family Asteraceae, comprises over 500 species distributed across temperate regions of Europe, Asia, and North America [7]. This genus is characterized by a diverse phytochemical composition, including terpenoids, flavonoids, coumarins, phenolic acids, and volatile oils, which confer multiple pharmacological properties [8]. Among its notable species, Artemisia annua is the natural source of artemisinin, the most effective treatment against malaria, for which its discoverers received the Nobel Prize in 2015 [9]. Artemisia absinthium (wormwood) has been traditionally used to treat digestive disorders, fever, inflammation, and respiratory ailments, and recent studies have demonstrated its role in managing immune-mediated conditions such as inflammatory bowel disease and nephropathy [11-10]. Meanwhile, Artemisia vulgaris (mugwort) is employed in Chinese and Indian medicine for gastrointestinal and menstrual disorders, and exhibits antioxidant, anti-inflammatory, and antimicrobial activities [12]. Other species, such as Artemisia californica, are used in traditional medicine to alleviate rheumatic pain, asthma, and menstrual disorders [13]. Nanotechnology-based materials have gained significant attention in recent years due to their potential applications in combating multidrug-resistant pathogens. Among these, iron oxide nanoparticles (IONPs) represent one of the most widely studied nanomaterials owing to their unique physicochemical properties, including superparamagnetism, high surface-to-volume ratio, and ease of surface modification [14] Several studies have demonstrated that IONPs exhibit potent antibacterial activity against both Gram-positive and Gram-negative bacteria, such as Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa [15] The underlying mechanisms involve the generation of reactive oxygen species (ROS), disruption of bacterial cell walls and membranes, leakage of intracellular contents, and interaction with nucleic acids and proteins, ultimately leading to cell death [16] Moreover, IONPs can enhance the efficacy of conventional antibiotics through synergistic effects, thereby providing a promising approach against multidrug-resistant strains [17] On the other hand, chitosan, a natural cationic polysaccharide derived from chitin, has been extensively studied for its biodegradability, biocompatibility, and antimicrobial properties [18]Its antibacterial activity is attributed mainly to the electrostatic interaction between its positively charged amino groups and negatively charged bacterial cell walls, leading to increased membrane permeability, leakage of intracellular constituents, and inhibition of DNA transcription In addition, chitosan and its nanoformulations have shown strong inhibitory effects on biofilm formation and virulence factors in multidrug-resistant pathogens, including MRSA and Klebsiella pneumoniae [19].

2. Materials and Methods

In this study, the following materials were used: ferric chloride (FeCl2 - 2H2O , 98%), anhydrous ferric chloride (FeCl3, NH4OH), NaOH, , CH3COOH ,Sod. Citrate and Chitosan was obtained from BDH, England. The media used for culture were (Mannitol Salt Agar, MacConkey Agar, Nutrient Broth, and Mueller-Hinton). Obtained from Liofilchem, USA.

Extraction Process

The Soxhlet apparatus was used for the extraction process in the Chemistry Laboratory - College of Education for Pure Sciences, University of Kirkuk, where 12 grams of the powdered plant model were placed inside the Thumble in the Soxhlet apparatus and using 250 ml of solvent (Ethanol 99%), at a rate of 6 hours of extraction per day, after which the extract was concentrated using a rotary evaporator at a temperature not exceeding 40°C. After evaporating all the solvent used in the extraction, a thick layer of the extract was observed to test its antibacterial effectiveness [20] .

Preparation of Iron Oxide Nanoparticles with Artemisinin

These materials used in the preparation of iron salts by the co-precipitation method are: We measure 0.69 g of iron (II) sulfate (FeSO4.7H2O) in 10 ml of ethanol 99% , and dissolve 1.35 g of iron (III) chloride (FeCl3.6H2O) in 10 ml of ethanol , the molar ratio is 1:2 , and add the artemisinin extract in a drip funnel dissolved in an alkaline solution, where we dissolve 1.77 g of sodium nitrate in 10 ml of ethanol and we dissolve 1.22 g of sodium hydroxide NaOH in 10 ml of ethanol in an oxygen-free environment containing only nitrogen gas N2[21].

Chitosan Coating

Chitosan polymer CS from Shaanxi Sangherb Bio-Techinc was used, at concentrations of (0.05, 0.100, 0.125, 0.150, 0.250) grams, and each concentration was dissolved in 30 ml of 1% dilute acetic acid (CH3COOH) solution [22].

Effect of Ethanol Extract and Iron Oxide Nanoparticles Coated with Chitosan on Bacterial Isolates

The disc diffusion method was used, as the test was conducted based on the method [23]. by transferring 3-5 pure colonies growing on the nutrient agar medium at the age of 24 hours to the nutrient broth medium, then incubating the medium at 37°C for 18-24 hours. The bacterial suspension was then diluted with normal saline solution, compared to the standard control tube, which is equivalent to (1.5 x 108) cells/ml. After the comparison, 0.1 cm of the bacterial suspension was withdrawn and spread on the surface of the Mueller-Hinton agar medium using a glass diffuser. The plates were then incubated in the incubator for 30 minutes to obtain absorption. To study the antibacterial activity of the plant extract and nanoparticles on the growth of bacteria, 6 mm diameter filter paper discs were prepared, saturated with different concentrations of the plant extract and nanoparticles to be tested. The discs were fixed with sterile forceps, and the plates were incubated at 37°C for 18-24 hours. An hour later, the inhibition zones were measured with a standard ruler.

3. Results and Discussion

Xrd Analysis Results

In Figure 1, we notice the appearance of peaks between angles 28.231° and 64.39°. The main peak is at angle 31.652° (367 counts, relative intensity 100%), indicating that the alcoholic extract contains diverse crystalline components. The sharper peaks at angles 28.231° and 31.733° indicate the presence of more regular crystalline materials, which are often the result of non-polar active compounds that ethanol prefers to extract. The crystal sizes range from 45 to 72 nm, indicating that the crystalline particles are small. In Figure 2, the XRD results showed the presence of distinct peaks that match the pattern of nano-iron oxide (Fe_3O_4 or γ-Fe_2O_3), indicating that the used nanomaterial maintained its crystalline structure after incorporation with the extract of the wormwood plant (Artemisia herba-alba). As for Figure 3, which represents (wormwood alcoholic extract + FeNPs + chitosan), we notice a decrease in the intensity of the Fe_3O_4 peaks, which indicates significant encapsulation or overlap with organic compounds. The highest percentage of crystal ambiguity (amorphous character) is due to the more diverse alcoholic components and their interaction with the particles, and this is consistent with [24].

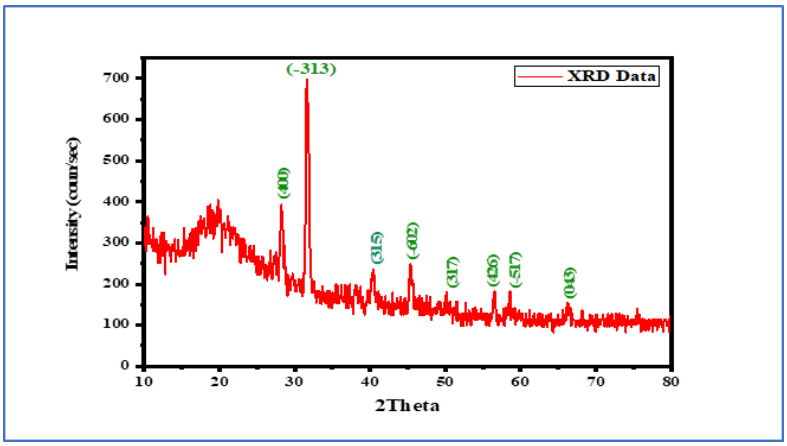

Figure 1. Results of X-ray diffraction examination of the ethanol extract of Artmesia.

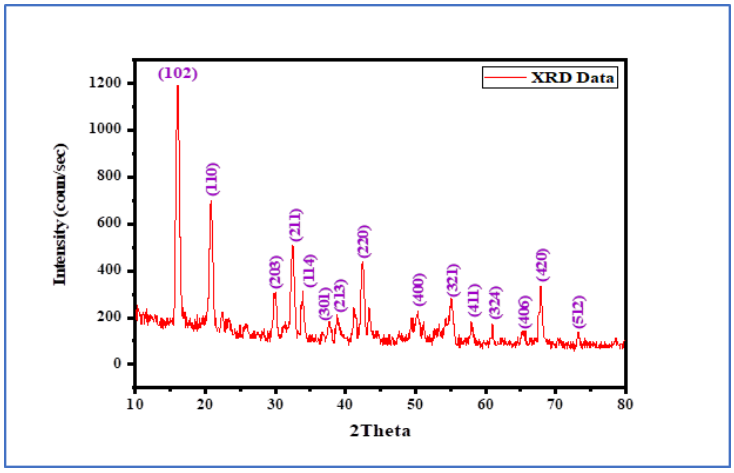

Figure 2. Results of X-ray diffraction of the ethanol of artmesia after adding Fe3O4 nanoparticles.

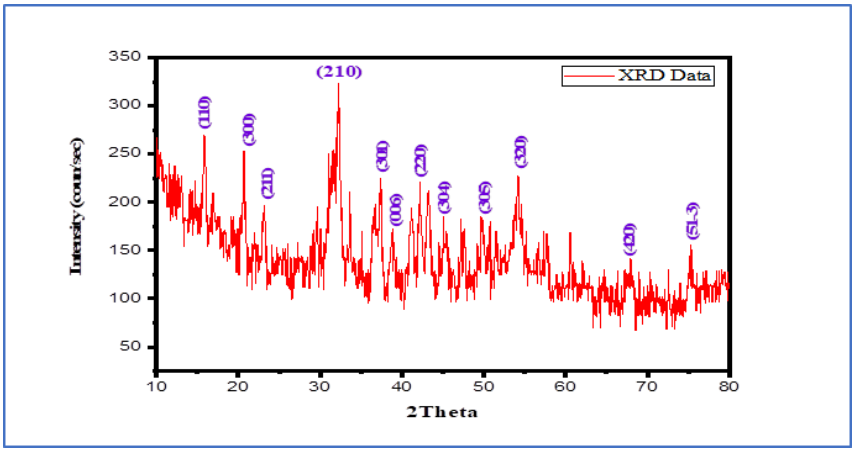

Figure 3. XRD results of ethanol extract of artmesia after adding Fe3O4 Nanoparticles with chitosan nanoparticles.

Scanning Electron Microscope (Sem)

Figure 4, which represents the alcoholic extract of wormwood, shows relatively smooth surfaces and a very homogeneous distribution. Alcohol as a solvent showed high efficiency in extracting organic compounds such as terpenes, resulting in a smooth, homogeneous structure . Figure 5 shows The wormwood extracts after adding iron oxide

particles (Fe_3O_4) show the formation of small spherical nanoparticles distributed on the surface of the extracts, and a clear interaction between the plant compounds and the nanoparticles appears, indicating the possibility of chemical or physical bonding and figure 6 show The wormwood extracts after adding (Fe_3O_4 + chitosan) show a network structure or a mixture of spherical clusters and fibrous surfaces, reflecting the polymeric nature of chitosan. These results are similar to the researcher's results [25].

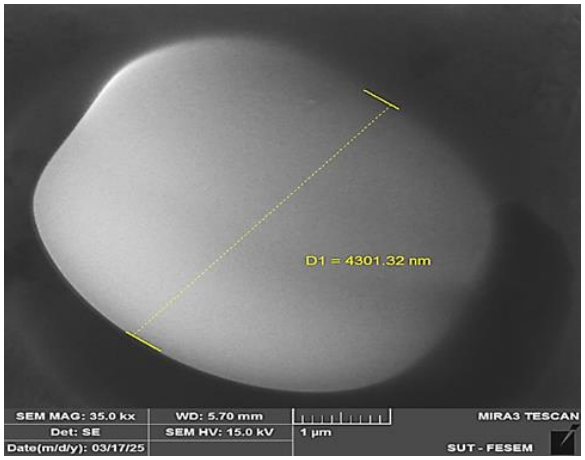

Figure 4. Is the result of SEM of the ethanol extract.

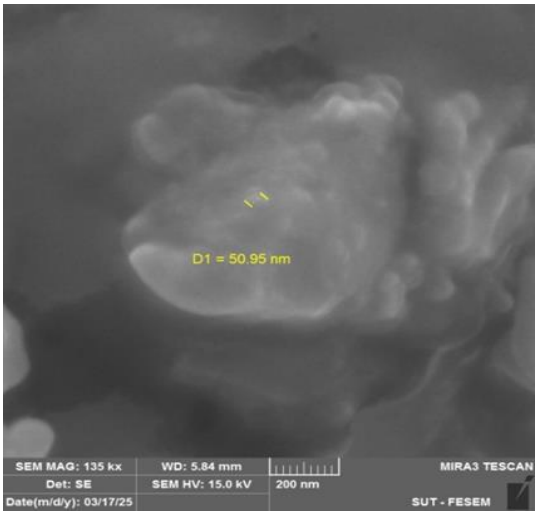

Figure 5. SEM result of the ethanol extract after adding Fe_3O_4

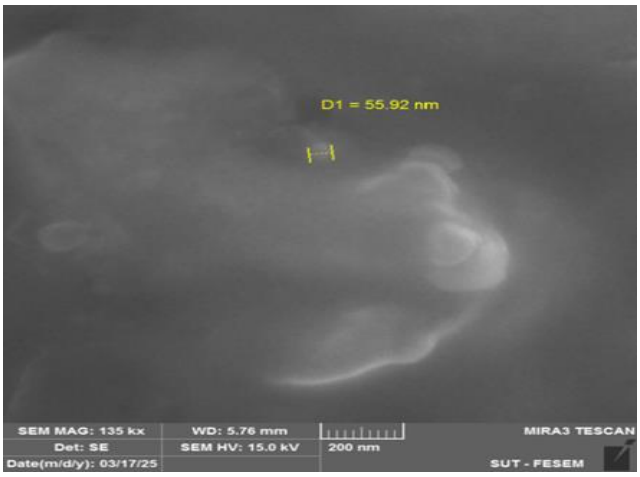

Figure 6. SEM result of the ethanol extract after adding (Fe3O4) coated with chitosan.

Transmission Electron Microscopy Result (Tem)

Figure 7, which represents the alcoholic extract of wormwood, shows relatively large or agglomerated particles. Alcohol extracts non-polar compounds (such as terpenes and essential oils) that may aggregate due to poor solubility in ethanol media. This is reflected in TEM as dark, irregular aggregates . Figure 8, which represents the alcoholic extract + Fe_3O_4, shows nanoparticles associated with heterogeneous organic compounds, with medium to large aggregates. The interaction of alcoholic compounds with Fe_3O_4 is less efficient than their ethanol counterparts, allowing the formation of irregular aggregates. This indicates the presence of partial agglomeration despite interaction with the plant material. Figure 9 represents the alcohol extract + Fe_3O_4 + chitosan. We notice the appearance of relatively larger particles and more pronounced aggregates compared to the hot sample. Alcohol extracts less polar plant compounds (such as terpenes and essential oils), which do not easily interact with Fe_3O_4 or chitosan. The weak interaction leads to the formation of an inhomogeneous layer around the particles, resulting in visible agglomerations in TEM. These results are similar to the researcher's results [25].

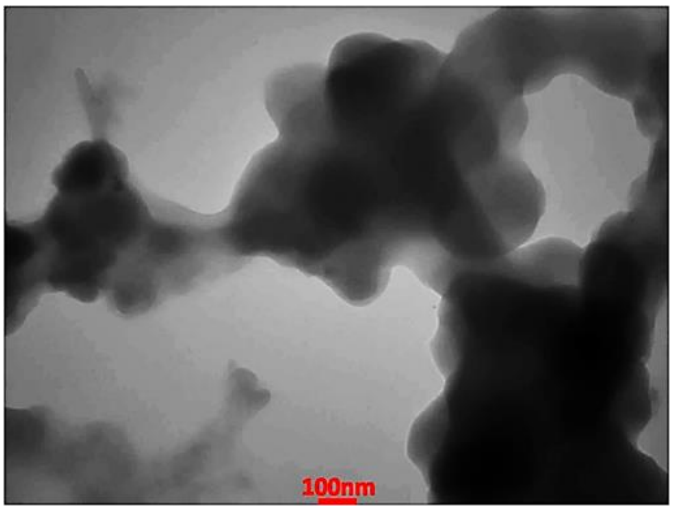

Figure 7. TEM image of the ethanol.

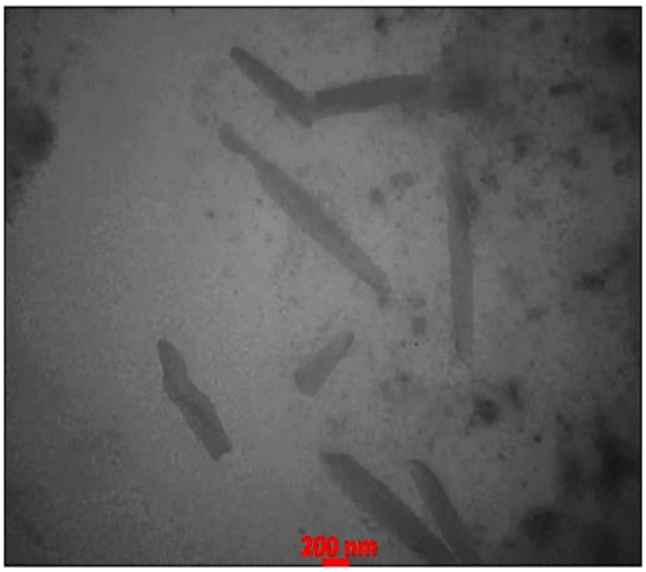

Figure 8. TEM result of the ethanol extract after adding (Fe3O4) nanoparticles.

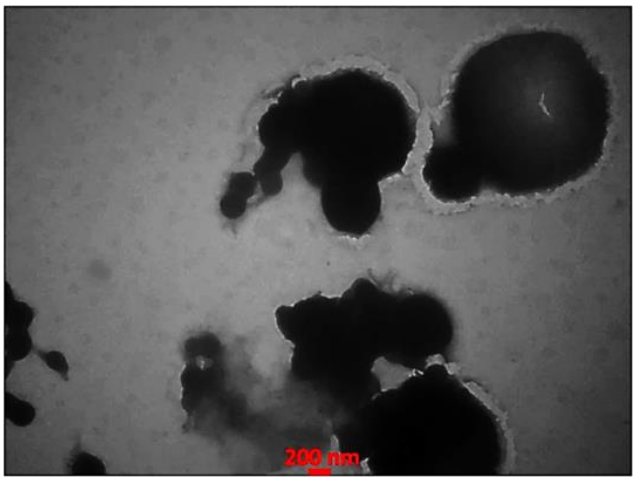

Figure 9. TEM result after adding (Fe3O4) with chitosan.

Zeta Sizer Results

The results showed that the alcoholic extract recorded a size value of 502.2 nm. The incorporation of magnetic nanoparticles (Fe_3O_4) led to significant changes in the particle size, as the alcoholic extract recorded an intermediate value (787.1 nm) due to the interaction of nanoparticles with relatively hydrophobic compounds, which led to larger aggregates than the hara-ma extract. After encapsulating them with chitosan, no significant stability was achieved, indicating the interference of organic solvents with the interaction of chitosan with Fe_3O_4.As shown in the figures 12-11-10.

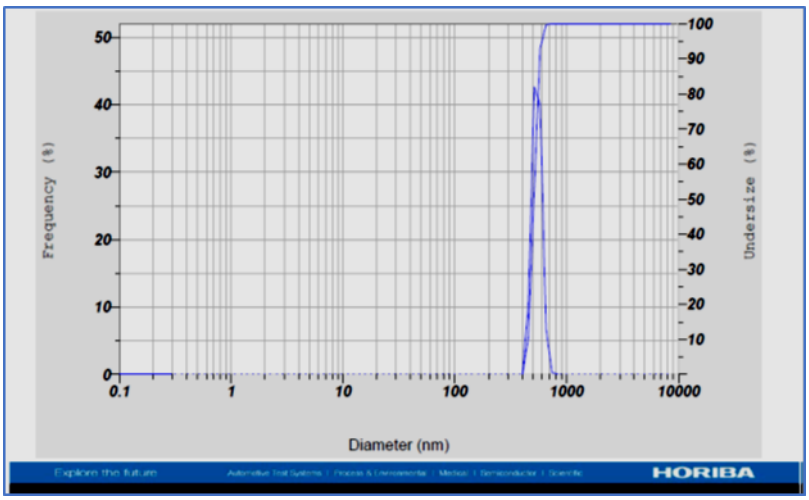

Figure 10. Zeta-sizer test results for the ehtanol extract.

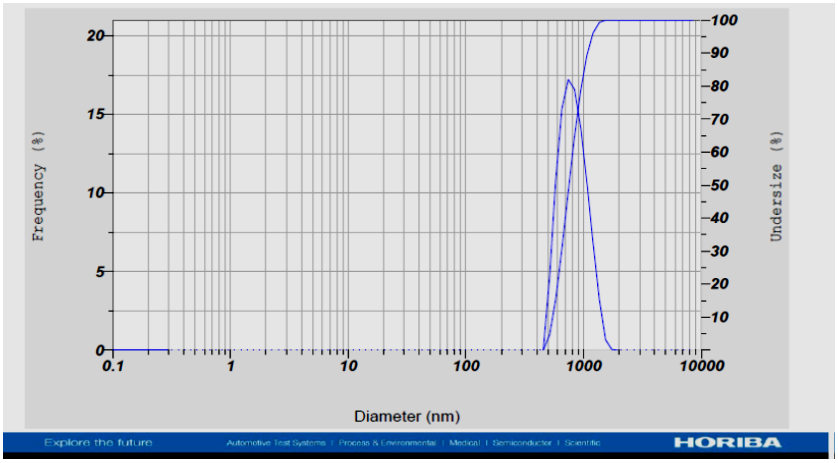

Figure 11. Zeta potential results for the extract after adding (Fe3O4) nanoparticles.

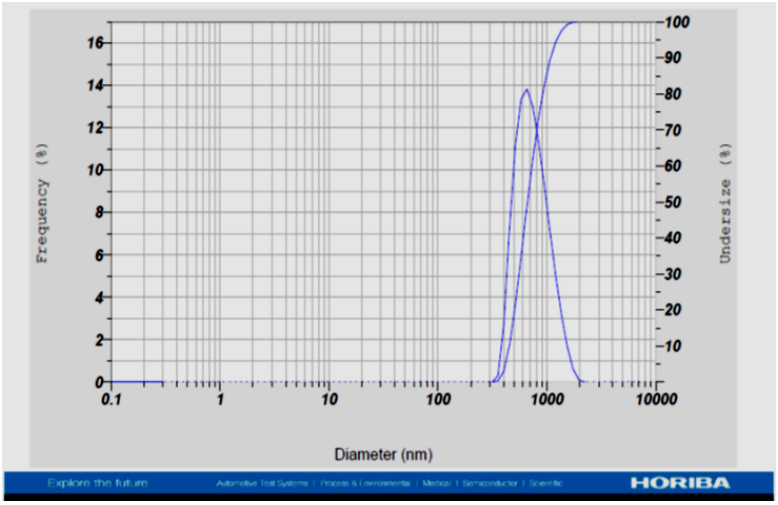

Figure 12. Zeta-Potential after adding iron oxide particles (Fe3O4) with chitosan.

ZETA Potential RESULTS

Zeta potential results for the plant extract Figure 13 showed that values were mostly -10 mV, which indicates poor to moderate stability in suspension. Alcoholic extracts tend to give less negative values due to the extraction of less polar compounds that affect the surface charge. In the pure state, the zeta potential of iron oxide nanoparticles (Fe_3O_4 or Fe_2O_3) Figure 14 can be positive or negative depending on the pH of the medium. When the particles are coated with a plant extract, the surface nature of the particles changes to that of the extract, causing a shift toward a negative charge. When the extract was loaded with Fe3O4 particles and chitosan nanoparticles Figure 15, a negative zeta potential was observed for this extract. Chitosan, in the pure state, exhibits a positive zeta potential due to the presence of amine groups ($–NH_3^+$), especially in acidic media.

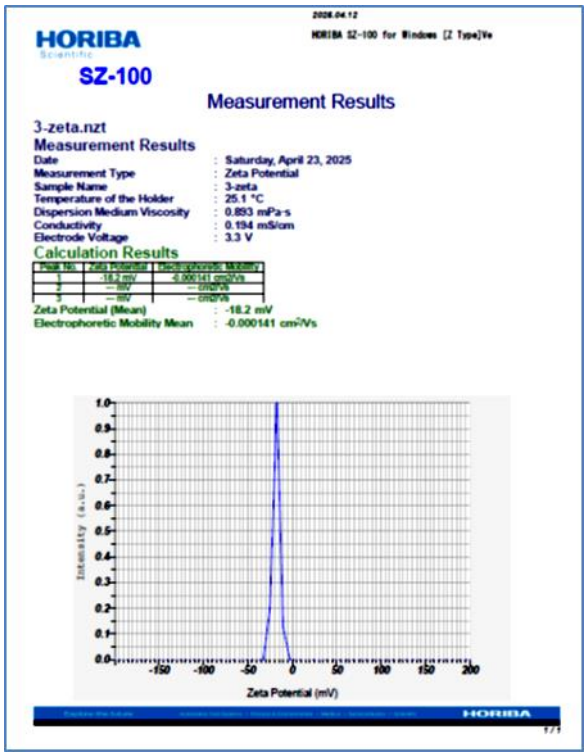

Figure 13. Zeta-potential test results for the ethanol extract.

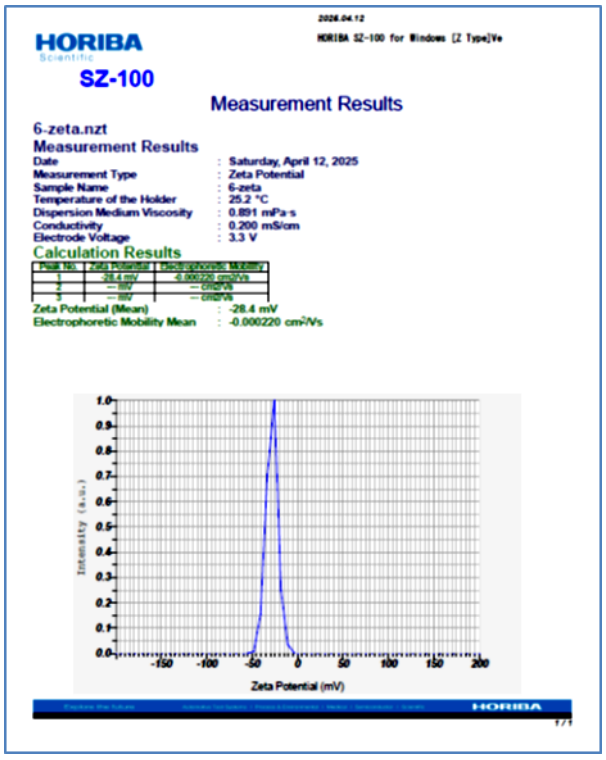

Figure 14. Zeta Potential resultst after adding (Fe3O4) nanoparticles.

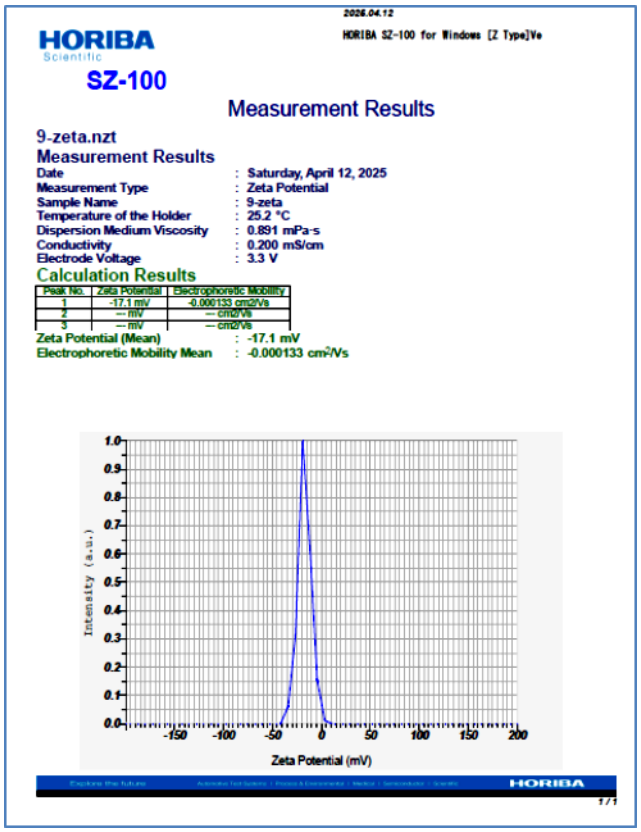

Figure 15. Zeta- Potential after adding iron oxide particles (Fe3O4) with chitosan.

The Effectiveness of Plant Extract and Nano Iron Oxide Coated with Chitosan

The results showed that the mean diameter of inhibition of the ehanol extract of wormwood against K. pneumoniae at concentrations of 350, 300, 250, and 200 mg/ml ranged from 26, 24, 22, and 20 mm, respectively. Meanwhile, the mean diameter of inhibition of S. aureus at the same concentrations was 18, 20, 23, and 25 mm. As for the

extract after adding iron oxide nanoparticles, the mean diameter of K. pneumoniae at concentrations of 350, 300, 250, and 200 μg/ml was 23, 24, 26, and 29 mm, while the mean diameter of S. aureus at the same concentrations was 20, 23, 25, and 28 mm. After coating with chitosan, the inhibition rates were higher at (35, 30, 28, 26) mm for K.pneumoniae and (34, 28, 26, 25) mm for S.aureus.

It has been observed that increasing the concentration of nanoparticles leads to higher inhibition rates, and their encapsulation with chitosan further enhances antibacterial efficacy. Iron oxide (Fe_3O_4) nanoparticles synthesized via the co-precipitation method have shown activity against both Gram-positive and Gram-negative bacteria, indicating their potential as effective antimicrobial agents [26].

The antibacterial mechanism is thought to involve electrostatic interactions, as the positively charged nanoparticles are attracted to negatively charged microbial surfaces. This interaction can induce oxidative damage, resulting in rapid microbial death. Typically, nanomaterials release metal ions that interact with thiol (–SH) groups in bacterial proteins, causing membrane disruption and cell lysis. Iron ions, in particular, can generate reactive oxygen species (ROS) such as hydroxyl radicals by converting hydrogen peroxide via the Fenton reaction. These radicals can damage bacterial polysaccharides, fragment DNA, and deactivate essential enzymes. Magnetic nanoparticles may also adhere to cell membranes or proteins, potentially impairing bacterial functions. Overall, oxidative stress mediated by ROS—including superoxide (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2)—is considered a major contributor to the antibacterial activity, with singlet oxygen ($^1O_2$) implicated in protein and DNA damage. The ROS generated by iron oxide nanoparticles have been shown to inhibit a wide range of pathogens, including Klebsiella pneumoniae and Staphylococcus aureus [27].

Coating iron oxide nanoparticles with chitosan significantly improves their physicochemical stability, biocompatibility, and biological performance, enhancing their utility in biomedical applications, particularly antimicrobial therapy. Chitosan, a natural polymer derived from chitin, is non-toxic, biodegradable, and highly compatible with biological tissues, reducing the likelihood of immune or cytotoxic responses during clinical use [28]. Moreover, chitosan prevents nanoparticle aggregation by acting as a stabilizing layer. Its positive charge and polymeric coating physically separate individual nanoparticles, minimizing van der Waals and magnetic interactions and thereby maintaining particle dispersion and stability [29].

4. Conclusion

The ethanol extract of Artemisia vulgaris showed notable antibacterial activity, which was further enhanced by the incorporation of Fe_3O_4 nanoparticles and chitosan coating. Physicochemical analyses (XRD, SEM, TEM, and Zeta potential) confirmed the successful synthesis of crystalline Fe_3O_4 nanoparticles, their stable dispersion, and the effective encapsulation by chitosan, which reduced aggregation and improved surface charge stability. From a microbiological perspective, the combined system exhibited significantly larger inhibition zones against Klebsiella pneumoniae and Staphylococcus aureus, indicating a synergistic effect between the plant bioactive compounds, the oxidative stress induced by Fe_3O_4 nanoparticles, and the membrane-disruptive action of chitosan. These results demonstrate that integrating nanomaterials with medicinal plant extracts provides a promising dual physicochemical and antimicrobial approach to combat multidrug-resistant bacteria.

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