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***“Salvia officinalis* Extract-Conjugated Magnetite and Selenium Nanocomposites Showed Enhanced Antibacterial and Anti-Biofilm Activity Against Multidrug-Resistant Pathogens”**

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Abstract

The increased prevalence of multidrug-resistant (MDR) pathogens and biofilm-associated infections underscores the urgent need for alternative antimicrobial strategies. In this study, MDR testing revealed lower resistance in Gram-positive bacteria (*Staphylococcus pasteurii*, *Listeria monocytogenes*, and *Bacillus cereus*; 69–77%) than in Gram-negative strains (*Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Escherichia coli*; 83–92%). Magnetite nanoparticles (Fe₃O₄NPs) and selenium nanoparticles (SeNPs) were synthesized via a green chemical reduction using L-ascorbic acid, followed by their conjugation with *Salvia officinalis* (SaO) aqueous extract to enhance stability and biological activities. TEM, DLS, XRD, and FT-IR analyses confirmed the nanoscale crystalline structure of the synthesized metal nanoparticles (MNPs) and the successful integration of SaO phytoconstituents into the nanocomposites. Phytochemical characterization further revealed variable adhesion and retention of SaO bioactive compounds within the two nanocomposites. The MIC values of SaO-Fe₃O₄NPs (0.03–1) and SaO-SeNPs (0.03–0.5 µg/mL) were not only dramatically lower than the SaO extract (125–500 µg/mL) and MNPs (0.5–25 µg/mL), but also comparable to or even exceeded meropenem (0.25–2 µg/mL). Vigorous antibacterial activity validated by TEM, revealing

extracellular and intracellular deformations, in accordance with the growth curve inhibition patterns. The nanocomposites also demonstrated potent antibiofilm activity, achieving 32.5–59.5% inhibition with SaO-Fe₃O₄NPs and 30.2–56.7% with SaO-SeNPs, surpassing meropenem, which exhibited maximum inhibition values of 32.5–47.6%.

Keywords: *Salvia officinalis*, metal nanoparticles, antibacterial activity, nanocomposites, MDR

Introduction

Multidrug-resistant (MDR) bacteria are the most significant global concern because they exhibit resistance to multiple conventional antibiotic classes and are frequently isolated worldwide **(1)**. This clearly demonstrated the necessity for innovative antimicrobial approaches **(1)**, such as plant extracts **(2)**, native or modified proteins **(3, 4)**, and nanoparticles **(5)**, which can be used alone or in combination with known antibiotics **(3, 6)**. Antimicrobial resistance (AMR), which underlies the development of MDR phenotypes, arises primarily from the misuse and overuse of antibiotics, such as prolonged or excessive dosing and administration without proper diagnosis, thereby posing a serious threat to human health **(7, 8)**. Conventional antibiotics typically target specific cellular sites; however, prolonged exposure can induce alterations in these targets, leading to

reduced drug uptake or failure of cell entry **(9)**. In addition, bacterial mutations may result in structural modifications of the cell wall or membrane, activation of efflux systems, and enzymatic inactivation of antibiotics, including the production of β -lactamases **(10)**. Consequently, the development of innovative antimicrobial agents with alternative and non-specific modes of action has become imperative.

Nanotechnology has emerged as a key field in developing advanced antibacterial agents **(11, 12)**. The synthesis of various nanomaterials has attracted considerable attention for their potential applications in imaging, diagnostics, and targeted drug delivery systems that enhance therapeutic efficiency **(13-15)**.

Among iron oxide nanomaterials, magnetite (Fe_3O_4), a magnetic iron oxide with an inverse spinel structure, possesses distinctive electrical and magnetic properties arising from electron transfer between Fe^{2+} and Fe^{3+} ions at octahedral sites **(16)**. Owing to their biocompatibility, low cytotoxicity, superparamagnetic, and high stability, Fe_3O_4 NPs have shown promise in drug delivery, hyperthermia, tissue engineering, and magnetic resonance imaging applications **(17, 18)**. Environmentally friendly routes for synthesizing iron nanoparticles have further advanced their biomedical relevance **(19)**.

Selenium has both pro- and anti-oxidative properties, making it a necessary trace element in the human body (approximately 40 μg Se/day). However,

large amounts of Se (around 400 $\mu\text{g day}^{-1}$) can be toxic and hazardous to health **(20, 21)**. Biological processes depend on selenium, which is also an essential element for antioxidant enzymes like glutathione peroxidases and thioredoxin reductases that shield the body from free radical species **(22)**. Selenium nanoparticles are known to be an essential trace element for human and animal health. They are necessary for the immune system to function correctly and to prevent the spread of illness **(23, 24)**. Because of their remarkable biological activity, low toxicity, and high bioavailability, selenium nanoparticles have attracted more attention in recent years for synthesis and analysis **(25, 26)**.

The medicinal plants have played an essential role in the treatment of numerous human diseases through their diverse bioactive compounds, which are commonly extracted from various plant parts, including leaves, fruits, stems, and roots, and are well recognized for their wide range of pharmacological properties, such as antiparasitic, antibacterial, antioxidant, antihypertensive, insecticidal, anticancer, antiviral, antifungal, and hypoglycemic activities **(27)**. *S. officinalis* L., an aromatic herb of the Lamiaceae family, is considered one of the most significant medicinal plants due to its diverse pharmacological activities and serves as a flavoring and aromatic agent in culinary preparations, as well as being widely employed in cosmetics and traditional medicine **(28)**. To maximize their therapeutic efficacy and facilitate practical applications, the development of advanced formulation technologies has become increasingly important **(29)**. Among

these strategies, the conjugation of plant extracts with nanoparticles has been widely investigated to improve biological stability, minimize toxicity, prolong bioactivity, and significantly strengthen their therapeutic potential **(27, 30, 31)**.

Biofilm-associated infections pose a significant challenge in clinical microbiology due to their elevated resistance to conventional antibiotics and the host's immune responses. The structural complexity of biofilms, including extracellular polymeric substances, limits drug penetration and promotes chronic infections **(32)**. Recently, plant extract-nanocomposites have shown promising antibiofilm potential through multiple mechanisms, including the generation of reactive oxygen species (ROS), disruption of quorum-sensing pathways, and inhibition of extracellular polymeric substance (EPS) production **(33, 34)**.

The present study aimed to evaluate the antimicrobial and antibiofilm activities of *S. officinalis* aqueous extract (SaO), metal nanoparticles (Fe₃O₄NPs, SeNPs), and their hybrid combinations against MDR Gram-positive and Gram-negative bacteria. Their effectiveness was further compared with that of conventional antibiotics to assess the potential of nanoparticle-based hybrids as sustainable and efficient alternatives for combating MDR and biofilm-associated infections.

Results

1. Antibiotic sensitivity of Gram-positive bacteria:

The antibiotic susceptibility of the tested Gram-positive bacteria was determined, and the results are given in **Table 1**. Meropenem was the only antibiotic that inhibited the growth of all three pathogenic strains (*S. pasteurii*, *B. cereus*, and *L. monocytogenes*), reflecting the highest antibiotic effectiveness (AE%) of 100%. Three antibiotics (azithromycin, chloramphenicol, and doxycycline) had a narrow antibacterial spectrum, inhibiting only one bacterium, namely *S. pasteurii*, and recorded 33% AE. Nine antibiotics, representing 69% of the total used (gentamicin, levofloxacin, nitrofurantoin, trimethoprim, clindamycin, linezolid, oxacillin, rifampin, and vancomycin), were totally ineffective, with 0% (AE%).

Alternatively, *B. cereus* and *L. monocytogenes* registered the highest multiple antibiotic resistance (MAR) index, 0.77; they could resist ten antibiotics among the thirteen total tested. This could be considered the highest resistance. Also, (*S. pasteurii*) exhibited a high MAR index, reaching a value of 0.69, which indicated resistance against nine antibiotics tested out of the total.

2. Antibiotic sensitivity of Gram-negative bacteria

As given in **Table 2**, meropenem was the only antibiotic that demonstrated measurable efficacy against all three Gram-negative pathogenic strains, achieving an AE% of 66%. In contrast, the remaining eleven antibiotics were completely ineffective, each recording an AE% of 0%.

The MAR index revealed that *E. coli* and *P. mirabilis* exhibited the highest resistance levels among the tested Gram-negative strains, with a MAR value of 0.92, indicating resistance to eleven out of twelve antibiotics. Likewise, *P. aeruginosa* showed a high MAR index of 0.83, corresponding to resistance to ten antibiotics.

3. Antibacterial activity of plant extract against Gram-positive and Gram-negative pathogens:

The antibacterial activity of SaO was studied, and the results are given in **Table S1**. At a concentration of 10000 µg/mL, SaO extract demonstrated a broad-spectrum antimicrobial effect, with inhibition zones ranging from 10.00 ± 0.26 mm to 15.00 ± 0.17 mm against both Gram-positive and Gram-negative bacteria. The most vigorous activity was observed against *S. pasteurii* (15.00 ± 0.17 mm) and *L. monocytogenes* (14.00 ± 0.26 mm), highlighting the higher susceptibility of Gram-positive strains. In contrast, Gram-negative bacteria such as *P. mirabilis*, *P. aeruginosa*, and *E. coli* exhibited more limited inhibition zones (10.00-12.00 mm). ANOVA analysis confirmed significant differences among the tested organisms ($P < 0.01$).

4. Characterization of Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs and SaO-SeNPs

The Fe₃O₄NPs demonstrated particle diameters in the range of 14.5–21.1 nm (mean: 18.69 ± 5.91 nm, median: 17.93 nm), exhibiting a characteristic dark contrast in TEM images due to the high electron density of iron oxide

(Fig. 1A). The Fe₃O₄NPs displayed a predominantly spherical morphology with smooth surface contours. While the SaO-Fe₃O₄NPs nanocomposites, TEM measurements revealed particle sizes spanning 8.7–25.4 nm (mean: 15.52 ± 4.36 nm, median: 15.16 nm) **(Fig. 1B)**. For SeNPs, TEM analysis revealed individual particle diameters ranging from 14.3 to 17.1 nm (mean $\pm$ standard deviation: 15.62 ± 4.62 nm, median: 14.69 nm, $n > 100$ particles) **(Fig. 2A)**. A notable increase in apparent particle size was observed in SaO-SeNPs, with measurements ranging from 17.25 to 40.70 nm (mean: 25.42 ± 7.12 nm, median: 24.65 nm) **(Fig. 2B)**. The dynamic light scattering (DLS) analysis was performed, and with results systematically compiled in **Table 3**, were 74.3 ± 4.5 nm for Fe₃O₄NPs **(Fig. 1C)** and 74.3 ± 4.2 nm for the SaO-Fe₃O₄NPs **(Fig. 1D)**, corresponding to 4.0-fold and 4.8-fold amplifications compared to TEM data. Likewise, DLS revealed the size of SeNPs was 69.4 ± 3.2 nm **(Fig. 2C)**, and the size of SaO-SeNPs was 69.4 ± 3.8 nm **(Fig. 2D)**, corresponding to 4.4-fold and 2.7-fold amplifications compared to TEM data. The SaO extract itself displayed a hydrodynamic diameter of 31.5 ± 2.1 nm. Polydispersity index (PDI) values are all below the threshold of 0.3, ranging from 0.155 to 0.238 across all nanoparticle samples. The lowest PDI value (0.155) was observed for SaO-Fe₃O₄NPs.

Zeta potential (ζ -potential) measurements were shown in **(Figs. 1E, 1F, 2E, 2F, and Table 4)**. All synthesized nanomaterials exhibited negative surface charges, with ζ -potential values ranging from -18.7 to -35.2 mV. Bare

nanoparticles ($\text{Fe}_3\text{O}_4\text{NPs}$ and SeNPs) exhibited moderate negative charges of -21.3 ± 1.8 mV and -28.5 ± 2.1 mV, respectively. A marked increase in surface negativity was observed in $\text{SaO-Fe}_3\text{O}_4$ NPs and SaO-SeNPs exhibiting ζ -potentials of -32.8 ± 2.6 mV and -35.2 ± 2.4 mV, respectively. Conductivity measurements ranged from 0.096 to 0.134 mS/cm, with pH values maintained in the near-neutral range (6.8–7.1).

The X-ray diffraction (XRD) pattern of $\text{Fe}_3\text{O}_4\text{NPs}$ resulted in intense diffraction peaks at $2\theta = 30.1^\circ, 35.5^\circ, 43.1^\circ, 53.5^\circ, 57.0^\circ$, and 62.7° (**Fig. 1G**), indexed to the (220), (311), (400), (422), (511), and (440) crystallographic planes, respectively, of the cubic spinel structure characteristic of magnetite (JCPDS 19-0629). The $\text{SaO-Fe}_3\text{O}_4\text{NPs}$ nanocomposite maintained the characteristic cubic spinel diffraction pattern with peaks at identical 2θ positions (**Fig. 1H**). Likewise, the XRD pattern of SeNPs with distinct diffraction peaks at 2θ values of $23.5^\circ, 29.7^\circ, 41.3^\circ, 43.7^\circ, 45.4^\circ, 51.8^\circ, 56.2^\circ$, and 61.7° (**Fig. 2G**), corresponding to the (100), (101), (110), (102), (111), (201), (112), and (202) crystallographic planes, respectively. Following the SaO-SeNPs nanocomposites, the XRD pattern retained the characteristic hexagonal selenium reflections at $2\theta = 23.6^\circ, 29.8^\circ, 41.4^\circ, 43.8^\circ, 45.5^\circ, 51.9^\circ, 56.3^\circ$, and 61.8° (**Fig. 2H**).

Fourier transform infrared spectroscopy (FT-IR) was acquired in the wavenumber range of $4000\text{--}400\text{ cm}^{-1}$ with 4 cm^{-1} resolution, and the results are compiled in (**Figs. 1I, 1J, and Figs. 2I, 2J**)

The Fe₃O₄NPs exhibited the O-H stretching band at 3435 cm⁻¹ and H-O-H bending at 1630 cm⁻¹. The diagnostic Fe-O vibrational modes of the spinel structure appeared as prominent absorption bands at 585 and 470 cm⁻¹. Similarly, SeNPs displayed a broad absorption band centered at approximately 3420 cm⁻¹. A distinct peak at 1635 cm⁻¹ was observed, while the absorption at 1080 cm⁻¹ was detected. An additional weak band at 680 cm⁻¹ was also observed. The FT-IR spectra of SaO-Fe₃O₄NPs and SaO-SeNPs revealed significantly enriched spectral profiles with additional absorption bands. Both conjugated systems exhibited a prominent absorption at 3410-3400 cm⁻¹, respectively. Distinct C-H stretching vibrations from aliphatic chains appeared at 2930-2925 cm⁻¹, respectively. A strong absorption band at 1705-1700 cm⁻¹, respectively, was observed. The appearance of aromatic C=C ring vibrations at 1615-1610 cm⁻¹, C-H bending modes at 1385-1380 cm⁻¹, and C-O stretching vibrations at 1240-1235 cm⁻¹ was also recorded. Aromatic C-H out-of-plane bending vibrations manifested as absorption bands at 815-810 cm⁻¹. Importantly, in SaO-Fe₃O₄NPs, the characteristic Fe-O vibrations remained evident (590 and 475 cm⁻¹).

5. Retention profile of phenolic and flavonoid compounds in nanocomposite substances

For phenolics, all six compounds initially present in the SaO extract were detected in the SaO-Fe₃O₄NPs with slight concentration variations. In contrast, SaO-SeNPs preserved only four phenolic compounds at lower

concentrations than both the SaO extract and SaO-Fe₃O₄NPs, except ferulic acid, which registered higher values than both. Regarding flavonoids, the SaO-FeNPs retained only five components out of seven, with minor differences compared with the crude extract. In contrast, SaO-SeNPs exhibited complete retention of all seven flavonoids, but in lower levels compared with SaO extract and SaO-FeNPs, as shown in **Table 5**.

6. Antibacterial activity of Fe₃O₄NPs and SeNPs

The antibacterial efficacy of both MNPs was studied. Results are given in **Table 6**.

At concentrations ranging from 5 to 250 µg/mL, Fe₃O₄NPs exhibited antibacterial activity that varied among the tested bacterial strains. Among Gram-positive bacteria, *L. monocytogenes* showed the highest overall susceptibility, with the largest mean inhibition zone (9.33 ± 3.19 mm), followed by *B. cereus* (7.00 ± 3.40 mm). The maximum inhibition zone against *L. monocytogenes* reached 16.00 ± 0.06 mm at 250 µg/mL. In contrast, Gram-negative bacteria demonstrated comparatively lower sensitivity to Fe₃O₄NPs, as reflected by smaller mean inhibition zones.

SeNPs also exhibit significant antibacterial activity. *L. monocytogenes* displayed the highest susceptibility, with an inhibition zone of 18.0 ± 0.06 mm at 250 µg/mL and a relatively high mean value of 7.67 ± 3.85 mm. Moderate sensitivity was observed for *P. mirabilis* and *P. aeruginosa* (14.00 ± 0.06 mm at 250 µg/mL), whereas *E. coli* exhibited the weakest response, with a maximum inhibition zone of 8.00 ± 0.12 mm and a mean of $3.33 \pm$

1.77 mm. *S. pasteurii* exhibited the most significant susceptibility to both MNPs in **Table 6x**. At lower concentrations (0.25-25 µg/mL), Fe₃O₄NPs showed non-significantly higher activity, reaching an inhibition zone of 25.0 ± 0.06 mm at 25 µg/mL, compared to 16.0 ± 0.06 mm recorded for SeNPs.

7. Antibacterial activity of SaO-Fe₃O₄NPs and SaO-SeNPs

The antibacterial efficacy of SaO-Fe₃O₄NPs and SaO-SeNPs against selected pathogenic Gram-positive and Gram-negative bacterial strains is shown in **Table 7**. At concentrations of 0.25-50 µg/mL, the highest antimicrobial effect was observed for both against *L. monocytogenes* (Gram-positive), with an inhibition zone of 20.0 ± 0.06 mm at 50 µg/mL. SaO-SeNPs demonstrated higher antibacterial activity against *B. cereus* (Gram-positive) and *P. mirabilis* (Gram-negative) than SaO-Fe₃O₄NPs. *P. aeruginosa* recorded slightly but significantly higher inhibition with SaO-Fe₃O₄NPs (mean: 6.57 ± 4.31 mm) than SaO-SeNPs (6.14 ± 3.48). Alternatively, *E. coli* remained the least affected by either treatment, showing limited zones of inhibition. SaO-Fe₃O₄NPs and SaO-SeNPs at very low concentrations (0.015 - 0.5 µg/mL) demonstrated the most potent antimicrobial effect against *S. pasteurii* (**Table 7x**), which showed the highest susceptibility. SaO-SeNPs demonstrated a greater non-significant inhibition zone (15.0 ± 0.06 mm) at a concentration of 0.5 mg/mL than SaO-FeNPs (8.00 ± 0.058 mm).

8. Comparative analysis of the prepared bioactive substances

As given in **Table 8**, the SaO extract displayed limited antimicrobial activity, with the lowest MIC (125 µg/mL) recorded against *S. pasteurii*. In comparison, *P. mirabilis* and *E. coli* showed high MIC values (500 µg/mL), while *L. monocytogenes*, *B. cereus*, and *P. aeruginosa* exhibited intermediate MIC values (250 µg/mL).

Fe₃O₄NPs and SeNPs exhibited markedly higher antimicrobial activity than the SaO extract. *S. pasteurii* was the most susceptible (MIC 0.5 µg/mL), whereas *B. cereus*, *P. aeruginosa*, and *E. coli* exhibited less susceptibility with high MIC values towards both MNPs (25 µg/mL). *L. monocytogenes* showed different susceptibility, being more susceptible to Fe₃O₄NPs (MIC 10 µg/mL) than SeNPs (MIC 25 µg/mL). *P. mirabilis* was the most susceptible to single MNPs (MIC 10 µg/mL) among Gram-negative bacteria.

SaO-Fe₃O₄NPs and SaO-SeNPs yielded the lowest MIC (0.03 µg/mL) against *S. pasteurii*, representing an approximately **17-fold** reduction compared with single MNPs. Moreover, MICs for *L. monocytogenes*, *B. cereus*, and *P. mirabilis* decreased by **10-50 fold by the combination**, against **5-50-fold** reductions in the case of *P. aeruginosa* and *E. coli*. Overall, the SaO-SeNPs consistently and significantly outperformed the SaO-Fe₃O₄NP against both Gram-positive and Gram-negative bacteria.

The SaO-SeNPs showed MIC values comparable to those of the reference antibiotic (meropenem) against Gram-positive bacteria but lower MICs against Gram-negative strains, indicating greater efficacy against the latter.

Conversely, the SaO-Fe₃O₄NPs exhibited relatively higher MIC values than meropenem in most strains, suggesting reduced potency against these strains, except for *P. mirabilis*, where both displayed equal MIC values. Interestingly, in the case of *S. pasteurii*, both SaO-Fe₃O₄NPs and SaO-SeNPs demonstrated markedly lower MIC values than meropenem.

9. Quantitative evaluation of growth inhibition of pathogenic bacteria by Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs, and SaO-SeNPs

Figs. 3 and 4 illustrate the 24h growth profiles of Gram-positive and Gram-negative bacteria at 2x MICs. Overall, bacterial growth decreased markedly compared to the control, with the SaO-nanocomposites exhibiting the most potent inhibitory effects.

The control culture typically reached maximum OD values (1.75–2.1) within 12–24 h. Among Gram-positive bacteria, *S. pasteurii* exhibited the highest sensitivity: Fe₃O₄NPs and SeNPs inhibited growth by 45% and 43%, respectively, while SaO-Fe₃O₄NPs and SaO-SeNPs further enhanced inhibition to 84% and 82%, respectively. Similar growth inhibition was observed in *L. monocytogenes*, with Fe₃O₄NPs and SeNPs suppressing growth by 43% and 42%, respectively, compared to 79% and 82% for their nanocomposites. For *B. cereus*, inhibition increased from 40% and 35% by Fe₃O₄NPs and SeNPs to 70% and 71% by SaO-Fe₃O₄NPs and SaO-SeNPs, respectively.

Regarding Gram-negative bacteria, Fe₃O₄NPs and SeNPs reduced the growth of *P. mirabilis* by 39% and 41 %, while SaO-Fe₃O₄NPs and SaO-

SeNPs recorded bigger growth reduction of 74% and 79%, respectively. For *P. aeruginosa*, Fe₃O₄NPs and SeNPs exhibited comparable inhibition, while the nanocomposites achieved 73% and 72% growth reductions, respectively. Fe₃O₄NPs and SeNPs induced 37% and 34% inhibition in the growth of *E. coli*, versus 69% and 68% in response to the SaO-Fe₃O₄NPs and SaO-SeNPs, respectively.

10. TEM images analysis of the sensitive bacterial cells

As shown in **Fig. 5**, TEM images revealed ultrastructural alterations in *S. pasteurii* (Gram-positive) and *P. mirabilis* (Gram-negative) treated with 2× MICs of the tested materials compared to the control. In the control, *S. pasteurii* appeared as intact cocci with dense, homogeneous cytoplasm and a clearly defined thick peptidoglycan layer. Likewise, *P. mirabilis* retained its typical rod-shaped morphology with well-preserved membranes. Cells of *S. pasteurii* and *P. mirabilis* exhibited clumped content (yellow arrows) and a non-homogeneous cell wall, indicating membrane disruption and cytoplasmic shrinkage (red arrows) induced by meropenem. In contrast, *S. pasteurii* cells treated with nanocomposites showed either severe shrinkage (red arrows) and rupture of the peptidoglycan layer (red arrows) with small cytoplasmic vacuolization (yellow arrows) in the case of SaO-Fe₃O₄, or extensive ultrastructure damage in the case of SaO-SeNPs.

P. mirabilis cells exhibited structural collapse, including outer membrane perforations and cytoplasmic disorganization in the case of SaO-Fe₃O₄. SaO-SeNPs treatment also caused pronounced deformation, including rupture of

the outer membrane, severe cytoplasmic disorganization, and vacuole-like empty spaces. The presence of black spots on or within the bacterial cells most likely corresponds to nanocomposite aggregates.

11. Quantitative antibiofilm activity of SaO, MNPs, and their nanocomposites compared with meropenem against the tested pathogenic bacteria.

The anti-biofilm activities of SaO extract, Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs, and SaO-SeNPs against pathogenic strains were compared with a reference antibiotic (meropenem).

As illustrated in **Fig. 6**, among all the tested strains, *S. pasteurii* showed the highest susceptibility to SaO-Fe₃O₄NPs and SaO-SeNPs; they achieved the highest inhibition extents (59.52 and 56.67%), respectively, surpassing their single MNPs (42.38 and 40.95%), the SaO extract (18.1%), and even the antibiotic meropenem (47.62%). Similarly, *L. monocytogenes* exhibited strong antibiofilm response to SaO-Fe₃O₄NPs and SaO-SeNPs, achieving biofilm reduction of 53.14% and 54.28%, respectively. Exceeding those recorded for their individual MNPs (41.71 and 40.57%), the SaO extract (16.57%), and meropenem (42.86%). *B. cereus* showed the least antibiofilm response within the Gram-positive bacteria group, while meropenem and the SaO extract demonstrated their usual inhibition extent, 42.21% and 15.58 %, respectively. However, the nanocomposites consistently achieved the highest inhibition of bacterial biofilm formation.

Generally, the Gram-negative bacteria exhibited lower biofilm inhibition than the Gram-positive strains. Among the tested Gram-negative bacteria, as shown in Fig. 7, *P. mirabilis* was the most responsive to the antibiofilm treatments, amounting to 52.36% and 54.45% with SaO-Fe₃O₄NPs and SaO-SeNPs, respectively, compared to 38.2% and 41.36% in the case of single MNPs, respectively. Meropenem displayed a moderate inhibition level of 41.36%.

Similarly, *P. aeruginosa* showed comparable sensitivity levels to the treatments. Both SaO-nanocomposites inhibited biofilm formation by approximately 51%, surpassing 12.1% for the SaO extract and about 35% for the meropenem. *E. coli* showed greater resistance, achieving the least inhibition in biofilm formation by the studied substances.

Discussion

In the present study, Gram-positive bacteria demonstrated higher sensitivity to the tested antibiotics than Gram-negative bacteria. This aligns with their structural composition, as Gram-positive bacteria possess a thick peptidoglycan layer but lack an outer membrane, facilitating the penetration and action of many antimicrobials (35). In contrast, the outer membrane of Gram-negative bacteria, enriched with lipopolysaccharides, acts as a selective barrier, limiting the uptake of several antibiotics (36, 37), including β -lactams, fluoroquinolones, glycopeptides, and even last-resort agents such as colistin (38). This intrinsic structural defense, combined with additional resistance mechanisms such as efflux pumps and

enzymatic inactivation, renders Gram-negative pathogens inherently more resistant than their Gram-positive counterparts **(10)**, which explains the notably high resistance rates observed in this study and reflects their MDR nature. Such widespread resistance is strongly linked to the excessive and inappropriate use of antibiotics in both human and veterinary medicine **(9)**. Meropenem, a carbapenem antibiotic, was the only agent that demonstrated broad-spectrum efficacy against both Gram-positive and Gram-negative bacteria. Carbapenems are unique among the currently available β -lactams due to their high stability against hydrolysis by most β -lactamases and their ability to act as slow substrates or inhibitors of these enzymes **(39)**. This property supports the present findings and suggests that the tested bacterial strains lacked efficient β -lactamase systems capable of inactivating meropenem. This activity is consistent with the well-established mode of action of meropenem **(40)**.

The enhanced biological activity of the SaO decoction extract may result from partial thermal hydrolysis of complex phytoconstituents into smaller derivatives, improving their permeability and bioavailability, consistent with previous reports **(41)**. At 10,000 $\mu\text{g/mL}$, the SaO extract exhibited moderate antibacterial activity, producing inhibition zones of 10–15 mm and showing greater efficacy against Gram-positive than Gram-negative bacteria. Similar findings have been reported for SaO extracts and their essential oils **(42, 43)**. The smaller inhibition zones compared with those of essential oils ($\approx$ 20–23 mm, **44**) likely reflect differences in the chemical composition and

concentrations of bioactive constituents. Overall, these findings highlight the selective antibacterial potential of SaO extract against Gram-positive pathogens and indicate that synergistic formulations may be required to improve efficacy against Gram-negative bacteria.

The slightly reduced mean size in the conjugated SaO-Fe₃O₄NPs, coupled with the presence of smaller nanoparticles (minimum 8.7 nm), suggests enhanced particle individualization and prevention of aggregation mediated by the organic stabilizing agents present in the SaO extract **(18)**. The size augmentation observed in SaO-SeNPs is particularly evident in certain particles approaching 40 nm, which is attributable to the organic coating layer derived from phytochemical constituents of the SaO extract, which forms a stabilizing shell around the SeNPs core. Moreover, the broader size distribution observed in SaO-SeNPs compared to bare SeNPs reflects the variable thickness of the organic capping layer, which depends on the differential adsorption kinetics of diverse phytochemical compounds present in the plant extract **(23)**. The hydrodynamic diameters measured by DLS were consistently larger than the corresponding TEM core sizes, a phenomenon attributed to the presence of hydration layers, surface-adsorbed biomolecules, and electrical double layers surrounding the nanoparticles in aqueous media and confirmed **(18)**. The DLS of the SaO extract confirms the colloidal nature of phytochemical aggregates present in the aqueous extract **(29, 34)**. The low Polydispersity index (PDI) values across all nanoparticles demonstrate relatively monodisperse colloidal

systems, typically considered acceptable for pharmaceutical applications, and validate the efficacy of the green synthesis approach in generating size-controlled nanomaterials with narrow distributions suitable for biomedical applications. The lowest PDI value (0.155) was observed for SaO-Fe₃O₄NPs, suggesting exceptional uniformity in the conjugated magnetite nanocomposite dispersion **(45)**. The negative ζ -potential values observed for all nanoparticle systems indicate the presence of surface-associated ionizable groups and suggest favorable dispersion behavior in aqueous media **(5)**. The Fe₃O₄NPs and SeNPs exhibited moderate surface charges ($|\zeta| > 20$ mV), indicating satisfactory colloidal stability **(33)**. Notably, conjugation with SaO extract significantly enhanced surface electronegativity, with ζ -potential values exceeding -30 mV. These elevated negative charges are indicative of excellent electrostatic stabilization and can be attributed to ionizable functional groups present in phytochemical constituents, particularly carboxylic acid moieties (-COO⁻) and phenolate groups (-O⁻) derived from polyphenolic compounds **(33, 34)**. Enhanced electronegativity not only promotes colloidal stability through intensified electrostatic repulsion but also modulates nanoparticle-biological interactions, potentially influencing cellular uptake mechanisms and biocompatibility profiles **(34)**. Furthermore, the low conductivity measurements confirm physiologically compatible suspension conditions suitable for biological applications. The XRD patterns, showing intense diffraction peaks, further verified the crystalline nature of both Fe₃O₄NPs

and SeNPs, consistent with previous reports on green-synthesized MNPs **(12, 17)**. The appearance of these reflections, particularly the strong (311) peak at 35.5° , serves as a diagnostic fingerprint for the inverse spinel structure (space group $Fd\bar{3}m$) of Fe_3O_4 **(16, 18)**. The $\text{SaO-Fe}_3\text{O}_4\text{NPs}$ nanocomposite maintained the characteristic cubic spinel diffraction pattern with peaks at identical 2θ positions, verifying the structural integrity of the magnetite core following SaO extract conjugation. **(16, 19)**. The diffraction peaks of SeNPs are in excellent agreement with the hexagonal selenium phase, confirming the formation of crystalline Se^0 during the green reduction process. The sharp, well-defined diffraction peaks indicate high crystallinity and minimal structural defects **(21)**. Notably, a slight broadening of diffraction peaks was observed in the conjugated system, attributable to the organic coating layer, which reduces the effective crystalline domain size detected by X-ray diffraction. This peak broadening phenomenon is consistent with the Scherrer equation relationship between crystallite size and diffraction peak width, and confirms the successful surface modification by organic phytochemicals without disrupting the underlying crystalline structure **(15, 16)**.

The FT-IR spectra of MNPs ($\text{Fe}_3\text{O}_4\text{NPs}$ and SeNPs) exhibited major peaks corresponding to the stretching vibrations of hydroxyl groups (O-H), originating from ascorbic acid used in their synthesis. This result confirmed the successful reduction of the metal precursors **(46)**. The diagnostic Fe-O vibrational modes of the spinel structure confirm the magnetite phase and

corroborate XRD findings. The higher-frequency Fe-O band (585 cm^{-1}) originates from Fe-O stretching in tetrahedral sites, while the lower-frequency band (470 cm^{-1}) corresponds to octahedral Fe-O vibrations, both characteristic of the inverse spinel crystal structure **(47)**. While the absorption at 1080 cm^{-1} indicated Se-O bonding, suggesting partial surface oxidation. An additional weak band at 680 cm^{-1} was assigned to Se-Se stretching vibrations characteristic of elemental selenium **(48)**. The FT-IR spectra of SaO-Fe₃O₄NPs and SaO-SeNPs showed additional absorption bands that were absent in the single MNPs; these new bands correspond to hydroxyl groups abundant in polyphenolic compounds present in SaO extract (e.g., caffeic acid, chlorogenic acid) **(19, 28, 48)**. The C-H stretching vibrations in both nanocomposites resulted from aliphatic chains. The C=O stretching of carboxylic acid functional groups (-COOH) is consistent with the presence of organic acids in the plant extract **(28)**. The appearance of aromatic C=C ring vibrations confirmed the presence of aromatic compounds, while C-H and C-O stretching further validated the organic coating **(30)**. In SaO-Fe₃O₄NPs, the characteristic Fe-O vibrations remained evident, demonstrating retention of the magnetite core structure beneath the organic shell. This comprehensive FT-IR analysis provides unequivocal molecular-level evidence that SaO phytochemicals, particularly polyphenolic compounds and carboxylic acids, have been successfully adsorbed onto nanoparticle surfaces through coordinate bonding and

electrostatic interactions, forming stable bio-conjugated nanocomposites with preserved inorganic cores and organic shells **(49)**.

Comparative phytochemical profiling revealed apparent differences between the SaO-Fe₃O₄NPs and SaO-SeNPs in terms of phenolic and flavonoid compounds retention. Interestingly, ferulic acid exhibited a higher concentration in SaO-SeNPs, suggesting a possible selective interaction with SeNPs that may stabilize or enhance its incorporation **(50)**. Such differential retention patterns may influence the overall bioactivity, as ferulic acid is well known for its antioxidant and antimicrobial roles **(51)**.

SaO-Fe₃O₄NPs maintained five of the seven compounds. Conversely, SaO-SeNPs exhibited complete retention of all seven flavonoids. This observation indicates that SeNPs may facilitate broad but less efficient entrapment of flavonoid compounds, while Fe₃O₄NPs provide more selective stabilization. Similar findings have been reported in other plant nanoparticle systems, where the type of metal precursor influenced the qualitative and quantitative retention of polyphenols **(52)**. These differences could partially explain the variations in antimicrobial and antibiofilm performance observed between SaO-Fe₃O₄NPs and SaO-SeNPs.

The antibacterial action of MNPs is evident due to their nanoscale size, as smaller particles increase the surface area-to-volume ratio and enhance interactions with microbial cell membranes **(53)**. However, their efficacy is moderated or limited at lower concentrations, particularly against Gram-negative bacteria, highlighting the need to combine or conjugate MNPs

with bioactive compounds or natural extracts to improve their antimicrobial performance. In this study, Fe₃O₄NPs and SeNPs exhibited distinct antibacterial activity against *S. pasteurii* (Gram-positive) at low concentrations (0.25–25 µg/mL), with no statistically significant difference between them. This similarity can be attributed to their overlapping particle size distributions, which likely minimized size-dependent effects. Moreover, nanoparticle size is not the sole determinant of antibacterial efficacy; other physicochemical factors, such as ion release, surface chemistry, and the ability to induce oxidative stress, play equally crucial roles **(54)**. The antibacterial activity of Fe₃O₄NPs observed in this study is consistent with previous reports demonstrating that iron oxide nanoparticles can generate ROS, which substantially contribute to their antibacterial activity **(55)**. The use of iron oxide nanoparticles is a promising way to overcome antimicrobial resistance because of their ability to interact with several biological molecules and to inhibit microbial growth. Iron oxide nanoparticles are defined as “nanozymes” due to their inherent enzyme-like activities and catalytic properties that are comparable to those of different oxidant systems, such as peroxidase and superoxide dismutase. Another feature of these NPs is their strong capacity to bind biological macromolecules, reflecting their function **(56)**.

The SaO extract in combination with MNPs could act synergistically, as the biological activity of the resulting SaO-nanocomposites was increased; this might be due to the presence of bioactive phenolic and flavonoid

compounds (e.g., caffeic acid, luteolin, apigenin, and quercetin derivatives), which possess numerous polar functional groups in combination with non-polar residues. Such residues, which could conjugate to MNPs with high electron density, might have greater capacity to disrupt bacterial cell membranes, thereby conferring greater antimicrobial and antibiofilm activity (57). Recent studies have shown that iron-based nanocomposites combined with antibiotics exhibit enhanced antimicrobial activity by exploiting multiple mechanisms, including ROS generation, membrane damage, and ion release, thereby overcoming bacterial resistance and synergistically improving antibiotic efficacy against MDR pathogens (58, 59). In contrast to surface-driven Fe-based antibacterial materials, the Fe_3O_4 and Se-based nanocomposites herein act at the nanoscale, exhibiting broader antimicrobial efficacy through the synergistic mechanisms discussed above (60).

The 24 h growth curves of six pathogenic bacteria exposed to 2x MICs of SaO-nanocomposites generally indicated more significant growth inhibition than MNPs, amounting to approximately 30-40% increases across all tested bacteria. **In Gram-positive** strains, this enhancement was particularly evident, which can be explained by their cell wall architecture (35), which allows nanoparticles and phytochemicals to penetrate the thick peptidoglycan layer and directly reach intracellular targets.

Although Gram-negative bacteria possess intrinsic resistance mechanisms (38), the SaO-nanocomposites still achieved significantly higher inhibition

than MNPs, which could be explained by the synergistic role of the SaO extract **(61)**.

TEM image of the *S. pasteurii* and *P. mirabilis* revealed shrinkage and instability of the bacterial cell wall by meropenem, which prominently targeted the cell wall in agreement with previous reports in this respect **(40)**.

In contrast, exposure of bacterial cells to SaO-Fe₃O₄NPs and SaO-SeNPs resulted in extensive cellular disruption, far exceeding that observed with meropenem. These findings agree with reports that magnetite and selenium nanoparticles generate ROS and disrupt thiol-rich proteins, thereby compromising membrane integrity and intracellular stability **(18, 22, 62)**. Among the tested nanocomposites, SaO-SeNPs had more impact on bacterial cells than SaO-Fe₃O₄NPs, which might be due to selenium's high reactivity toward thiol-containing proteins, leading to oxidative stress and enzyme inhibition. **(22, 63)**.

Further work will be necessary to study synergism between MNPs and SaO extract and their applications in vitro and in vivo; the work in this respect is still under investigation.

Conclusion

This study demonstrates that conjugating the SaO extract with Fe₃O₄NPs and SeNPs yields SaO-nanocomposites with remarkable antibacterial and anti-biofilm efficacies, effectively addressing the urgent challenge of multidrug resistance. The composites exhibited MIC values significantly

lower than those of the SaO extract and individual MNPs, and, in several cases, superior to those of the reference antibiotic meropenem. Their antibiofilm inhibition also exceeded that of meropenem, underscoring their potential as next-generation antimicrobial agents. Comprehensive physicochemical characterization confirmed successful synthesis and retention of bioactive phytochemicals within the nanocomposites, while TEM analysis validated profound structural damage in bacterial cells. Collectively, these findings highlight the synergistic interplay between phytochemicals and nanoparticles, offering a promising strategy to combat MDR pathogens and biofilm-associated infections. Future investigations should extend to in vivo assessments and mechanistic studies to advance their translation into clinical applications.

Material and Methods

1. Bacterial strains and media:

The bacterial pathogens used in this study were preserved as frozen stocks at -20°C in glass beads. Before examination, they were revived and cultured in Brain Heart Infusion broth (BHI) (Oxoid, UK). The test panel included three Gram-positive bacteria, viz *L. monocytogenes* LMG10470, *B. cereus* ATCC14579, and *S. pasteurii* (64), along with three Gram-negative bacteria, viz *P. mirabilis* DSM4479, *E. coli* LMG8223, and *P. aeruginosa* LMG8029. All bacterial strains were obtained from the Culture Collection of the Botany and Microbiology Department, Faculty of Science, Zagazig University,

Egypt. After propagation in BHI broth, the strains were maintained on BHI agar slants at 4°C and sub-cultured monthly onto fresh BHI agar (Oxoid, UK).

2. Antibiotic susceptibility test (AST)

Antibiotic susceptibility testing for experimental strains was performed as described previously **(65)**. Bacterial inoculum was adjusted to match the turbidity of a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL. The standardized suspensions were applied onto Mueller-Hinton agar plates (Oxoid, UK) using a sterile automatic pipette (Promega, USA) and evenly spread with sterile cotton swabs. For Gram-negative bacteria, twelve antibiotics were tested, while thirteen antibiotics were applied for Gram-positive bacteria, as presented in **Tables 1 and 2**. Antibiotic disks were placed on the agar medium surface using sterile forceps, ensuring adequate spacing between disks to prevent overlapping of inhibition zones. The plates were incubated at 35°C for 24–48 h. After incubation, inhibition zone diameters were measured using a millimeter ruler. The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024) **(66)**, and bacterial sensitivity or resistance was classified following **(67)**.

3. Plant Material

Dried SaO leaves were purchased from a local market in Zagazig, Sharkia, Egypt. The plant leaves were identified by Dr. Samir Salem, from the

Botany and Microbiology Department, Faculty of Science, Zagazig University, Egypt.

3.1. Preparation of SaO extract (decoction method)

The aqueous extract of SaO was prepared by decoction as described previously **(68)** with minor modifications. Briefly, 10 g of dried SaO leaves were ground into a fine powder using a laboratory blender and boiled in 100 mL of distilled water for 20 min. The mixture was then cooled to room temperature and filtered through Whatman No. 1 filter paper. The resulting filtrate was collected and stored at 4°C until used.

4. Green synthesis of MNPs using L-ascorbic acid

The synthesis of Fe₃O₄NPs and SeNPs was carried out via chemical reduction using L-ascorbic acid as both a reducing and capping agent **(69)**. Aqueous solutions of 1.34g FeCl₂·4H₂O and 3.40g FeCl₃·6H₂O for Fe₃O₄NPs, and 50 mM sodium selenite (Na₂SeO₃) for SeNPs, were each dissolved separately in 60 mL of deionized water. The mixture was heated to 80°C for magnetite and 65°C for selenium under vigorous magnetic stirring until the solution became homogeneous and no visible solid residues were observed. Then, an aqueous solution of L-ascorbic acid (50 mM) was added dropwise to each precursor solution. The reaction mixtures were then adjusted to an alkaline pH (10) by the dropwise addition of 5 mL of 1% NaOH. The resulting mixtures were further stirred for 1h, during which a gradual color change to dark brown for Fe₃O₄NPs and reddish orange for SeNPs occurred. Finally, the deionized water was added to the reaction mixtures to

reach a final concentration of 1000 $\mu\text{g/mL}$. After overnight incubation at room temperature, the mixtures were subjected to ultrasonic treatment (sonication) for 30 min at a temperature of 35 $^{\circ}\text{C}$ to enhance nanoparticle dispersion and prevent agglomeration. The resulting SeNPs and $\text{Fe}_3\text{O}_4\text{NPs}$ were collected by centrifugation, washed repeatedly with distilled water, and stored at 4 $^{\circ}\text{C}$ until used

5. Preparation of SaO- $\text{Fe}_3\text{O}_4\text{NPs}$ and SaO-SeNPs

The SaO-nanocomposites were prepared by combining green-synthesized magnetite and selenium nanoparticles with SaO aqueous extract at a 1:1 (w/w) ratio, following a modified method (70). After MNPs preparation, 0.1 g of dried SaO extract was dissolved in 100 mL of distilled water to obtain a 1000 $\mu\text{g/mL}$ stock solution. Subsequently, 50 mL of the extract solution was added to 50 mL of each MNPs suspension under continuous stirring. Upon mixing, the SaO- $\text{Fe}_3\text{O}_4\text{NPs}$ developed a dark brown color approaching black, while the SaO-SeNPs exhibited a deep orange to reddish hue, confirming a successful complex formation. The resulting mixtures were then sonicated at 35 $^{\circ}\text{C}$ for 30 min to enhance homogeneity and stabilize the interaction between the MNPs and SaO extract. The final suspensions were cooled to room temperature and stored in dark containers at 4 $^{\circ}\text{C}$ for further characterization. The nanocomposite concentration was adjusted to 1000 $\mu\text{g/mL}$.

5.1. Characterization of $\text{Fe}_3\text{O}_4\text{NPs}$, SeNPs, SaO- $\text{Fe}_3\text{O}_4\text{NPs}$ and SaO-SeNPs

5.1.1. Transmission electron microscope (TEM)

The morphology and size of the Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs, and SaO-SeNPs were characterized by TEM operated at 100 kV connected with a CCD camera, Japan **(71, 72)**.

5.1.2. Dynamic light scattering (DLS) and zeta potential

The DLS was employed to determine the hydrodynamic diameter, size distribution profile, and surface charge and stability of MNPs and SaO-nanocomposites suspended in solution **(73)**. Zeta potential was conducted to assess nanoparticle surface charge and predict colloidal stability in aqueous suspension. The measurements were performed using the Malvern Zetasizer system (Malvern Instruments, UK) at the Regional Center for Food and Feed, Agricultural Research Center (ARC), Giza, Egypt.

5.1.3. X-ray diffraction (XRD)

X-ray diffraction analysis was carried out using a Bruker D8 Discover diffractometer (Billerica, MA, USA) to investigate the crystalline structure, confirm the successful formation, and assess the homogeneity and purity of MNPs and SaO-nanocomposites **(74)**, carried out at the Agricultural Research Center (ARC), Giza, Egypt.

5.1.4. Fourier transform infrared spectroscopy (FT-IR)

The characterization of the functional groups on the surfaces of the synthesized MNPs and SaO-nanocomposites was analyzed by FT-IR (Thermo Nicolet model 6700 spectrometer, Micro-Analytical Center, Cairo

University, Giza, Egypt). The measurements were carried out using the KBr pellet method, and spectra were recorded in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} **(75)**.

6. High-performance liquid chromatography (HPLC) for MNPs and SaO-nanocomposites

Analysis of phenolic and flavonoid compounds was performed using an HPLC system (Agilent Series 1100, Agilent, USA), equipped with an auto-sampling injector, solvent degasser, two LC pumps (Series 1100), UV/V detector, and ChemStation software. Detection was carried out at 250 nm for phenolic acids and 360 nm for flavonoids using a C18 column (125 mm $\times$ 4.6 mm, 5 μm particle size). Phenolic acids were separated using a gradient mobile phase consisting of solvent A (methanol) and solvent B (acetic acid in water, 1:25 v/v). The gradient program started with 100% (B) for 3 minutes, followed by 50% (A) for 5 minutes. The concentration of A was then increased to 80% for 2 minutes before returning to 50% (A) for an additional 5 minutes. Flavonoids were separated using an isocratic elution with acetonitrile (A) and 0.2% (v/v) aqueous formic acid (B) at a 70:30 (A: B) ratio. The solvent flow rate was 1 mL/min, the column temperature was maintained at 25 $^{\circ}\text{C}$, and the injection volume was 25 μL for both analyses **(76, 77)**.

7. Screening for the antibacterial activity of the SaO extract, MNPs, and their nanocomposites against tested bacteria using agar well diffusion assay:

The antibacterial activity of SaO, Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs, and SaO-SeNPs was studied against indicator bacteria: Gram-positive *S. pasteurii*, *B. cereus*, and *L. monocytogenes*, as well as Gram-negative *P. mirabilis*, *P. aeruginosa*, and *E. coli* through the agar well diffusion assay, as reported by Balouiri (67). The Mueller-Hinton agar plate surface was inoculated with overnight bacterial suspensions 1.5×10^8 CFU/mL (0.5 McFarland standard). Wells of 7 mm diameter were then aseptically punched using a sterile micropipette tip at suitable spaces from each other, and 50 μ L of each antimicrobial sample at graded concentrations was carefully loaded into the wells. The plates were incubated at 35 °C for 24 h, and the inhibition zone diameters (mm) were measured with a millimeter ruler.

7.1. Determination of minimum inhibitory concentrations (MICs) of the SaO, MNPs, and their nanocomposites against tested bacteria by microdilution assay:

The MIC values of SaO, MNPs, and their nanocomposites were determined using the broth microdilution method in sterile 96-well microplates, with the lowest concentration that completely inhibited visible bacterial growth after overnight incubation. (78) Approximately 1×10^6 CFU/mL of each bacterial inoculum was added to BHI broth containing serial two-fold dilutions of the

SaO (125-500 $\mu\text{g/mL}$) or MNPs (0.25-250 $\mu\text{g/mL}$) or their nanocomposites (0.015-50 $\mu\text{g/mL}$), mixed in equal volumes (1:1v/v).

After incubation at 37 °C for 18 h, bacterial growth was assessed by measuring the optical density at 600 nm (OD 600) using a microplate reader (Bio-Rad 680 XR, Hertfordshire, UK). All assays were performed in triplicate, and mean values were recorded for statistical analysis.

7.2. Bacterial growth curve (turbidity test)

Aliquots (50 μL) of the previously prepared bacterial cultures of suspension containing about 1×10^6 CFU/mL, incubated at 37°C for 4 h in BHI broth, were transferred into the wells of a sterile 96-well microplate. Bacterial cells were then treated with 50 μL of 2x MICs of either the MNPs or the SaO-nanocomposites. The negative control contained only BHI broth, while the positive control contained untreated bacterial cultures.

Bacterial growth was monitored by OD at 600 nm using a microplate reader (Bio-Rad 680XR, Hertfordshire, UK) **(79)**. Turbidity readings were recorded at 0, 4, 6, 12, 18, and 24 h of incubation, and OD values were used to construct bacterial growth curves for each treatment.

7.3. Transmission electron microscope (TEM)

S. pasteurii (Gram-positive) and *P. mirabilis* (Gram-negative) were selected for TEM analysis because they exhibited the highest sensitivity to the tested treatments, particularly SaO-Fe₃O₄NPs and SaO-SeNPs. The tested bacterial strains were cultivated in BHI broth and treated with 2×MIC concentrations of the SaO-nanocomposites or meropenem (positive control).

Untreated bacterial suspensions served as the negative control. After incubation at 37 °C for 4h, the treated and control bacterial cells were harvested, fixed, and examined as described previously (80).

8. Evaluation of anti-biofilm activity of the SaO extract, Fe₃O₄NPs, SeNPs, SaO-Fe₃O₄NPs, and SaO-SeNPs compared with meropenem

The anti-biofilm activity was evaluated as described previously (81, 82) using the microtiter plate crystal violet assay. The assay was performed to assess the inhibitory effect of each treatment on biofilm formation by the tested bacterial strains. 100 µL of the bacterial inoculum was standardized to approximately 1×10^6 CFU/mL in Tryptic Soy Broth (TSB), followed by the addition of 100 µL of the tested compound at 2×MIC concentrations. Plates were incubated at 37 °C for 24 h under static conditions. After incubation, wells were gently washed three times with sterile phosphate-buffered saline (PBS) to remove planktonic cells. The remaining attached biofilms were fixed with methanol, stained with 0.1% crystal violet for 15 min, and then the excess stain was rinsed off. The bound dye was solubilized using 200 µL of 95% ethanol, and the (OD) was measured at 570 nm using a microplate reader.

9. Statistical Analysis

The data were analyzed using analysis of variance (ANOVA) to evaluate the effects of different elements with different concentrations on the inhibition zone diameters of the tested bacteria. Mean values and standard errors

(SE) were calculated for each treatment. ANOVA was performed to determine the significance of the factors studied and their interaction. When significant differences were detected ($P < 0.01$), mean comparisons were carried out using the least significant differences (LSD) test. All statistical analyses were performed using GenStat 18th edition. Data are presented as means $\pm$ standard error (SE). The figures were constructed using Excel 2021.

Declarations

Abbreviations

BHI: Brain Heart Infusion

AST: Antibiotic susceptibility test

CLSI: Clinical and Laboratory Standards Institute

MDR: Multidrug-resistant

AE%: Antibiotic effectiveness percentage

SaO: *Salvia officinalis* L.

MNPs: Metal nanoparticles

MIC: Minimum inhibitory concentration

Fe₃O₄NPs: Magnetite nanoparticles

SeNPs: Selenium nanoparticles

HPLC: High-performance liquid chromatography

DLS: Dynamic light scattering

XRD: X-ray diffraction

FT-IR: Fourier transform infrared spectroscopy

TEM: Transmission electron microscope

Ethics approval and consent from the participant

The study did not involve animal or human experimentation.

Consent for publication

All participating authors agreed on the publication of the current article.

Availability of Data and Materials

Data will be made available on request.

Competing interests

The authors declare no competing interests.

Authorship contributions

GE: Conceptualization, Resources, Writing – review & editing, Supervision.

NA: Experimentation, writing, analysis, review, editing, and validation. MM:

validation, analysis, investigation, preparation. AO: Conceptualization,

Methodology & Investigation. SA: Resources, Writing – review & editing,

Supervision. MS: Conceptualization, Resources, Writing – review & editing, Supervision.

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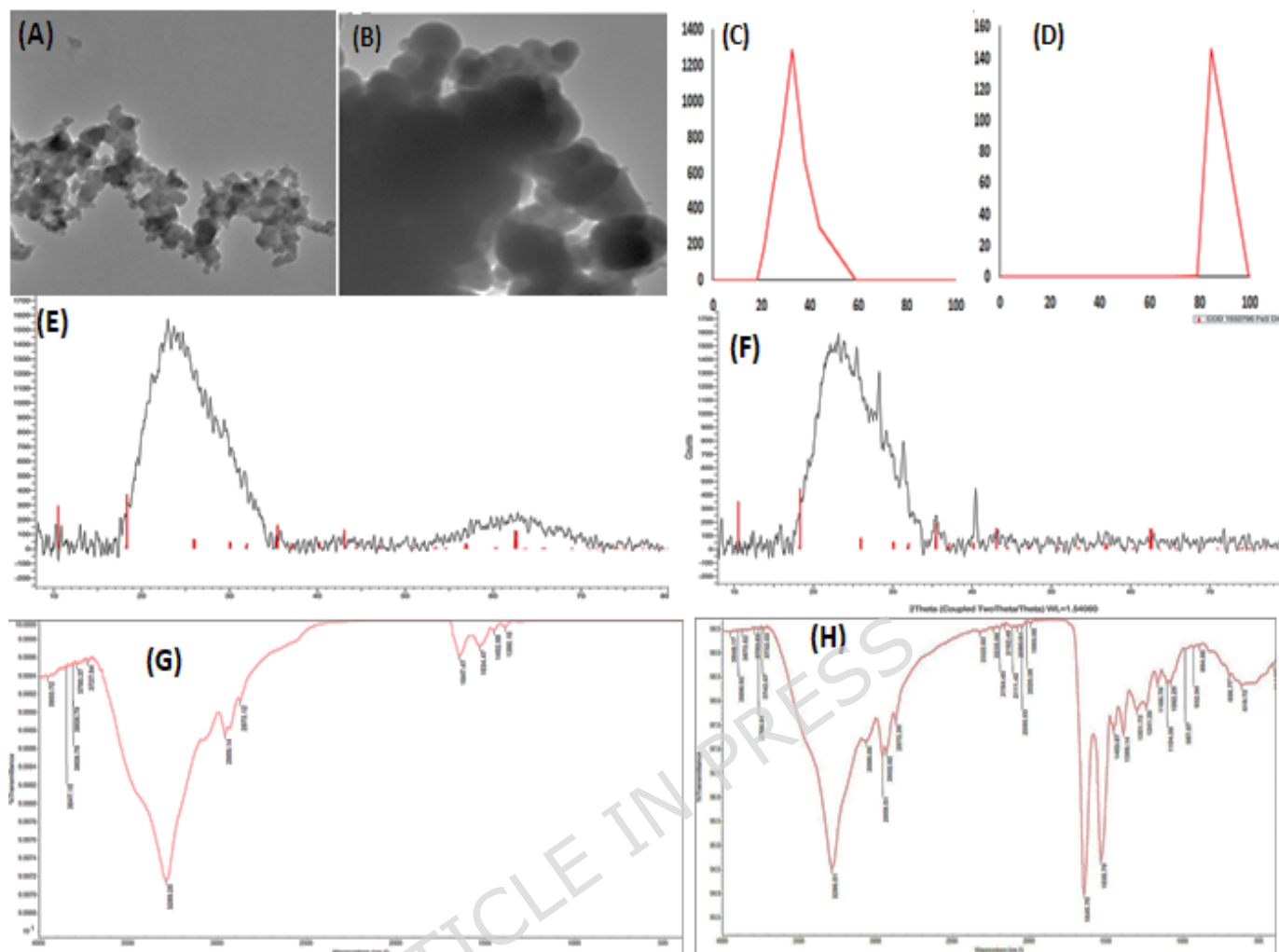


Fig.1. Characterization of magnetite nanoparticles: (A) TEM image of single Fe₃O₄NPs; (B) TEM image of conjugated SaO-Fe₃O₄NPs; (C) DLS particle size distribution of Fe₃O₄NPs; (D) DLS particle size distribution of SaO-Fe₃O₄NPs; (E) XRD of Fe₃O₄NPs; (F) XRD of SaO-Fe₃O₄NPs; (G) FTIR of Fe₃O₄NPs; and (H) FTIR of SaO-Fe₃O₄NPs.

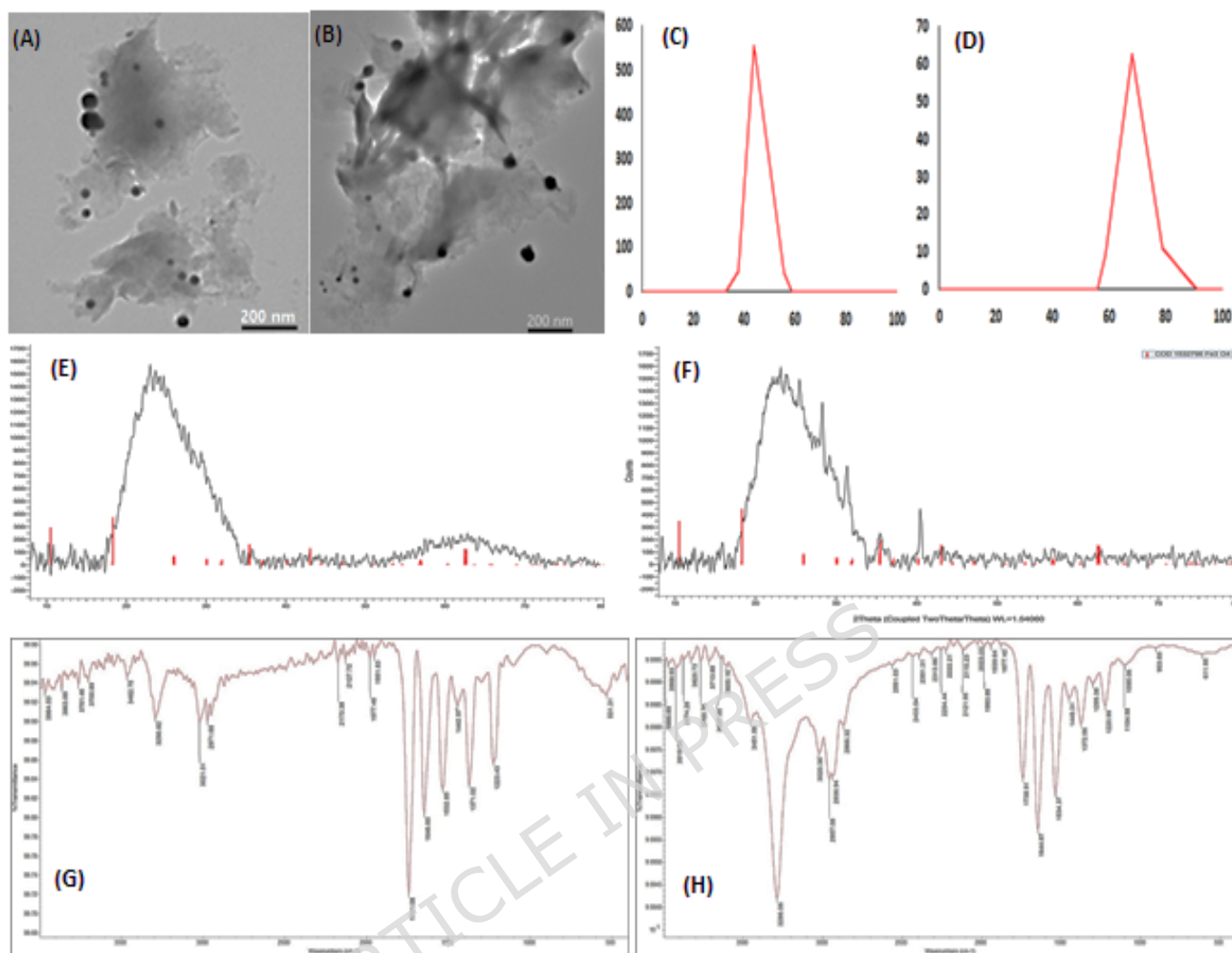


Fig.2. Characterization of selenium nanoparticles: (A) TEM image of single SeNPs; (B) TEM image of conjugated SaO-SeNPs; (C) DLS particle size distribution of SeNPs; (D) DLS particle size distribution of SaO-SeNPs; (E) XRD of SeNPs; (F) XRD of SaO-SeNPs; (G) FTIR of SeNPs; and (H) FTIR of SaO-SeNPs.

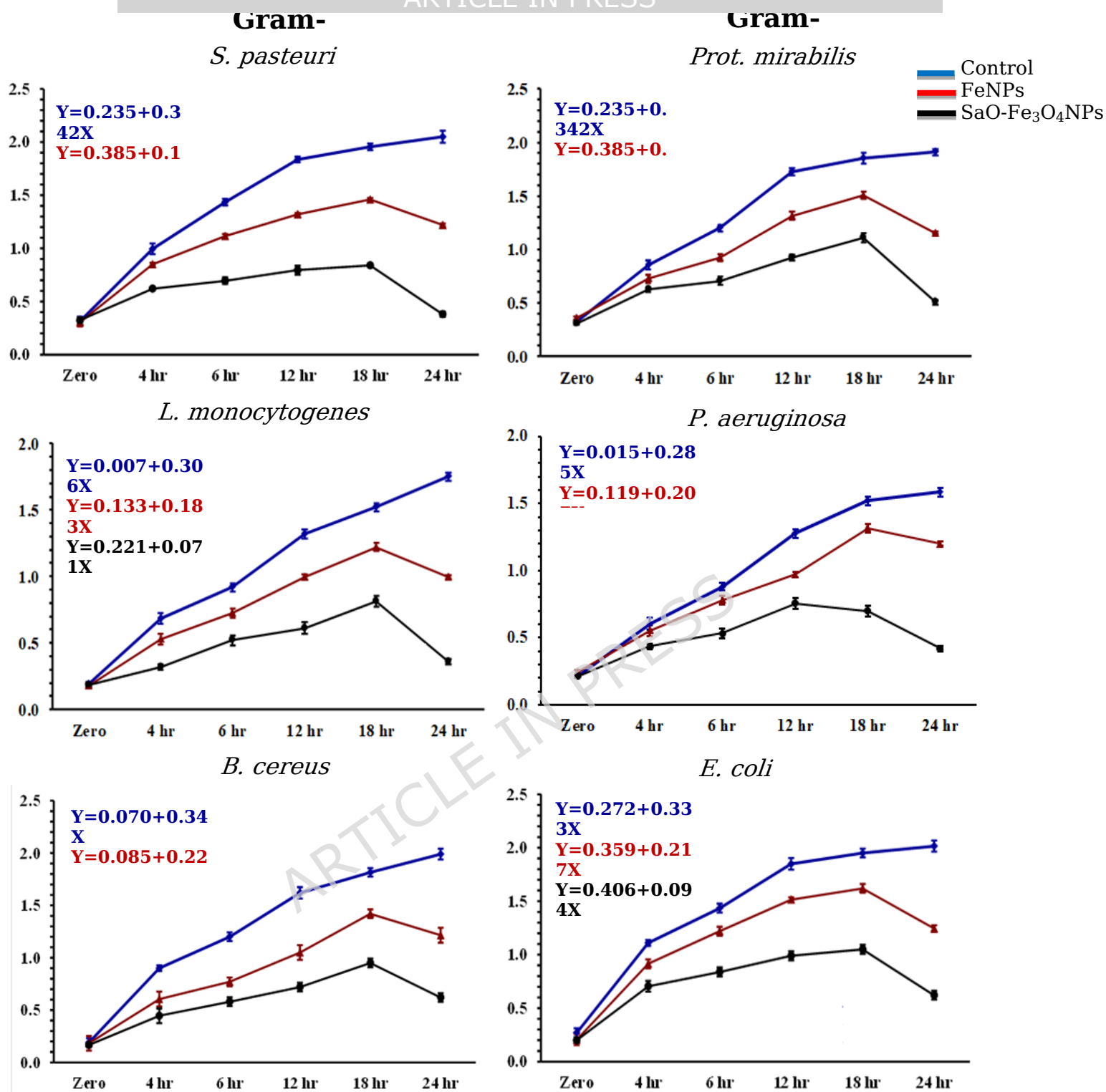


Fig.3. Quantitative inhibition profile of pathogenic bacteria exposed to magnetite nanoparticles (Fe₃O₄NPs) and their conjugated form (SaO-Fe₃O₄NPs).

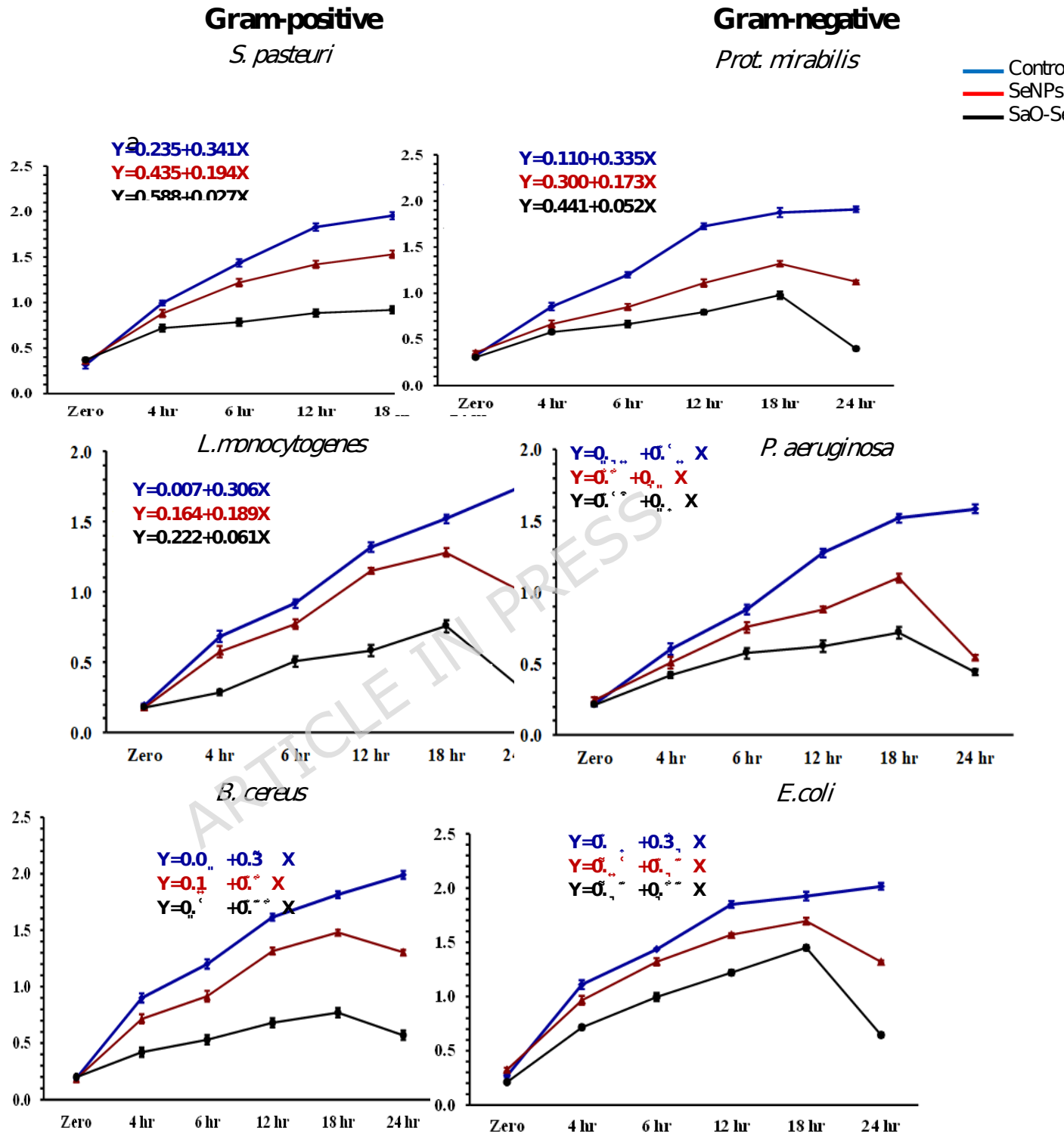
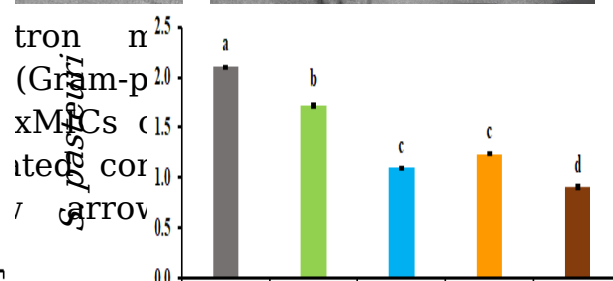
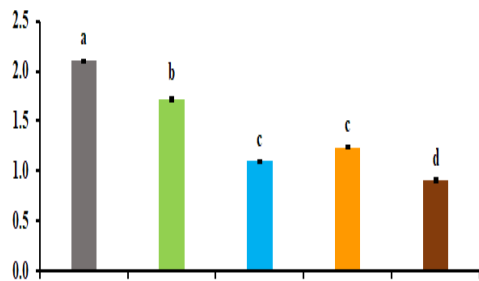
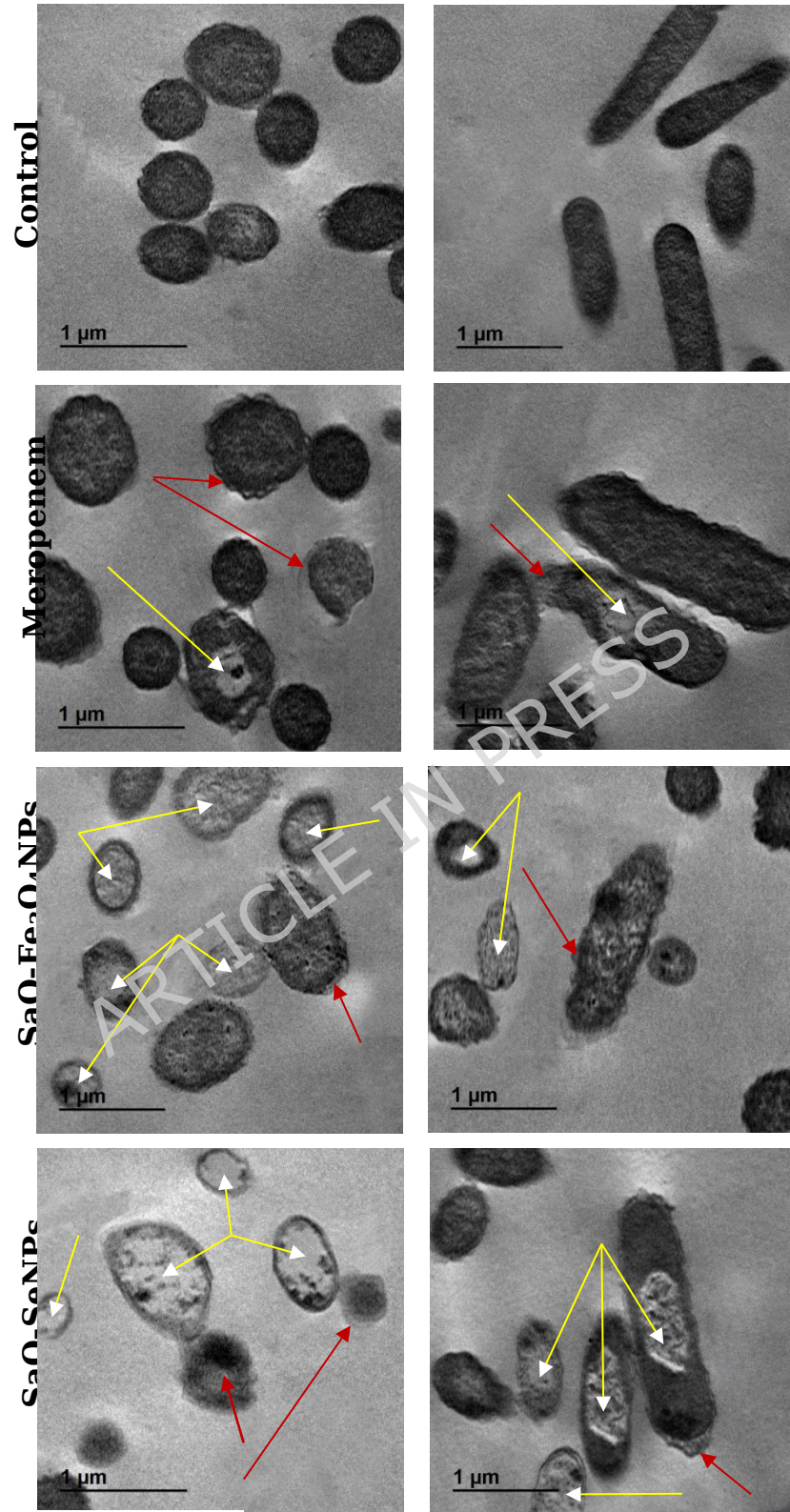


Fig4. Quantitative inhibition profile of pathogenic bacteria exposed to selenium nanoparticles (SeNPs) and their conjugated form (SaO-SeNPs).

Staphylococcus pasteuri *Proteus mirabilis*



TEM images of *Staphylococcus pasteuri* (Gram-positive) and *Proteus mirabilis* (Gram-negative) under different conditions: Control, Meropenem, SaO-Fe₃O₄-NPs, and SaO-SaNDs. The images show the morphology of the bacteria and the presence of extracellular material. The scale bar represents 1 μm.

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Fig. 6. Showing comparative antibiofilm activity of *Salvia officinalis* L. extract (SaO), MNPs ($\text{Fe}_3\text{O}_4\text{NPs}$ and SeNPs), and their composites ($\text{SaO-Fe}_3\text{O}_4\text{NPs}$, SaO-SeNPs) against three Gram-positive bacteria relative to meropenem.

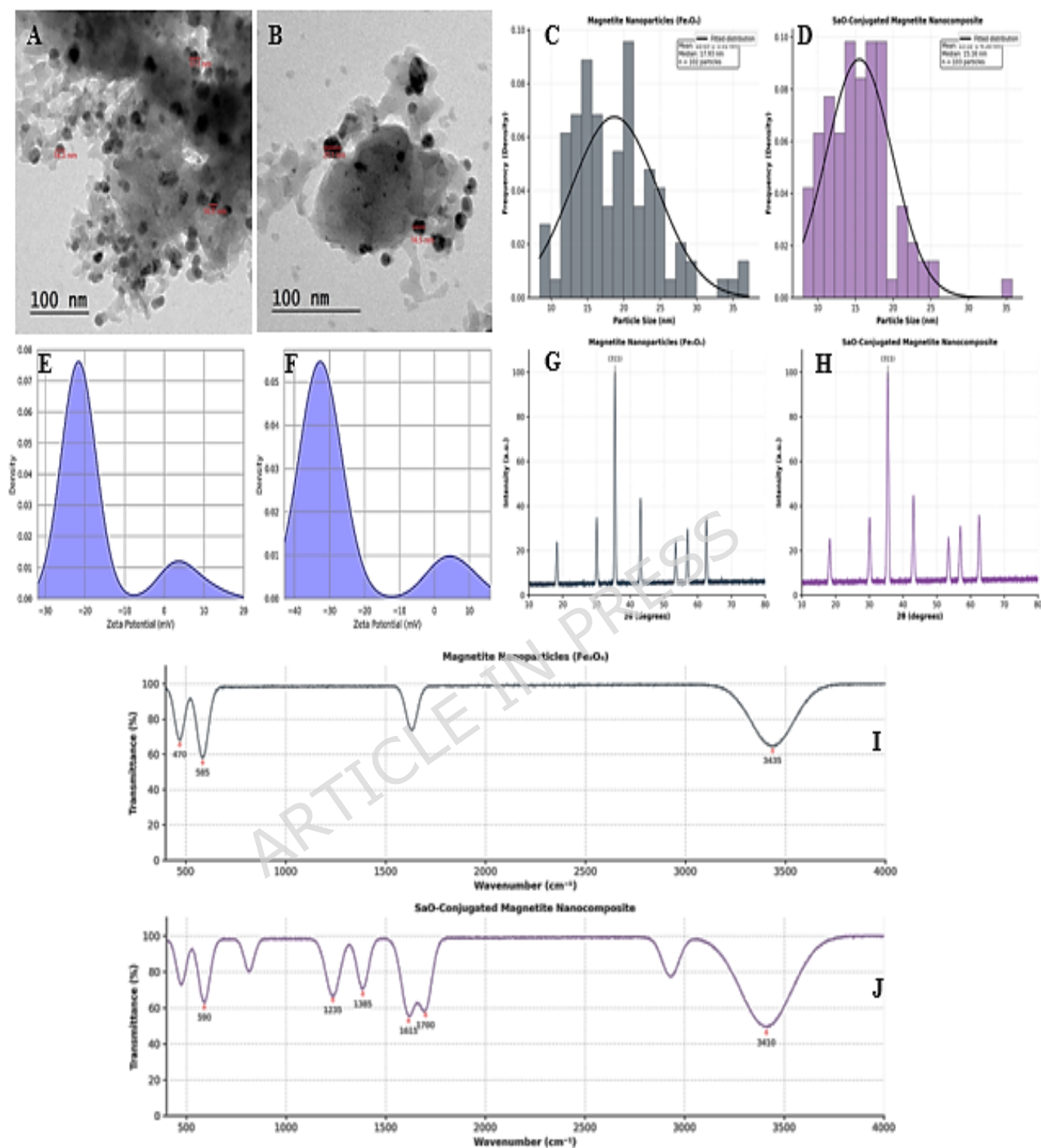


Fig.1. Characterization of magnetite nanoparticles ($\text{Fe}_3\text{O}_4\text{NPs}$): **(A)** TEM image of $\text{Fe}_3\text{O}_4\text{NPs}$; **(B)** TEM image of conjugated SaO- $\text{Fe}_3\text{O}_4\text{NPs}$; **(C)** DLS particle size distribution of $\text{Fe}_3\text{O}_4\text{NPs}$; **(D)** DLS particle size distribution of SaO- $\text{Fe}_3\text{O}_4\text{NPs}$; **(E)** Zeta potential of $\text{Fe}_3\text{O}_4\text{NPs}$; **(F)** Zeta potential of SaO- $\text{Fe}_3\text{O}_4\text{NPs}$; **(G)** XRD of $\text{Fe}_3\text{O}_4\text{NPs}$; **(H)** XRD of SaO- $\text{Fe}_3\text{O}_4\text{NPs}$; **(I)** FTIR of $\text{Fe}_3\text{O}_4\text{NPs}$; and **(J)** FTIR of SaO- $\text{Fe}_3\text{O}_4\text{NPs}$.

Table 1. Antibiotic sensitivity of Gram-positive bacteria.

Antibiotics	Category & inhibition zone (mm) ^b		Classification of Tested bacteria (S-R-I) according to the inhibition zone (mm) ^c			% Antibiotic effectiveness (AE) ^d
	S	R	<i>S. pasteuri</i>	<i>B. cereus</i>	<i>L. monocytogenes</i>	
Azithromycin (15µg)	(≥18)	(≤13)	S (25)	R (6)	R (8)	33%
Chloramphenicol (30µg)	(≥18)	(≤12)	S (25)	I (15)	R (14)	33%
Doxycycline (30µg)	(≥19)	(≤14)	S (27)	R (14)	R (12)	33%
Gentamicin (10µg)	(≥15)	(≤12)	R (10)	R (12)	R (14)	0%
Levofloxacin (5µg)	(≥21)	(≤15)	R (20)	R (20)	I (18)	0%
Nitrofurantoin (300µg)	(≥17)	(≤14)	R (12)	R (10)	R (14)	0%
Trimethoprim (5µg)	(≥16)	(≤10)	R (10)	R (0)	R (0)	0%
Clindamycin (2µg)	(≥21)	(≤14)	R (12)	R (14)	I (18)	0%
Linezolid (30µg)	(≥21)	(≤20)	R (18)	R (16)	R (10)	0%
Oxacillin (30µg)	(≥22)	(≤21)	R (0)	R (0)	R (0)	0%
Rifampin (5µg)	(≥20)	(≤16)	R (0)	R (0)	R (0)	0%
Vancomycin (30µg)	(≥12)	(≤16)	R (9)	I (14)	R (5)	0%

Meropenem (10µg)	(≥2 3)	(≤1 9)	S (30)	S (27)	S (28)	100%
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^a (The Clinical & Laboratory Standards Institute).

^b (M100 consult CLSI M100 ED30, 2021).

^c S, I & R (Sensitive, intermediate & resistance).

^d AE Antibiotic effectiveness % = (No of Sensitive strain/total No of strain).

^f Multiple antibiotic resistance (MAR) index = a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

MAR INDEX **0.69** **0.77** **0.77**

Table 2. Antibiotic sensitivity of Gram-negative bacteria.

Antibiotics	Category & inhibition zone (mm) ^b		Classification of Tested bacteria (S-R-I) according to the Inhibition zone (mm) ^c			% Antibiotic effectiveness (AE) ^d
	S	R	<i>E. coli</i>	<i>Prot. mirabilis</i>	<i>P. aeruginosa</i>	
Azithromycin (15µg)	(≥18)	(≤1 3)	R (8)	R (8)	R (8)	0%
Chloramphenicol (30µg)	(≥18)	(≤1 2)	R (12)	R (12)	R (10)	0%
Doxycycline (30µg)	(≥19)	(≤1 4)	R (12)	R (13)	R (12)	0%
Gentamicin (10µg)	(≥15)	(≤1 2)	R (12)	R (8)	I (14)	0%
Levofloxacin (5µg)	(≥21)	(≤1 5)	R (15)	R (14)	R (6)	0%
Nitrofurantoin (300µg)	(≥17)	(≤1 4)	R (8)	R (0)	R (0)	0%
Trimethoprim (5µg)	(≥16)	(≤1 0)	R (0)	R (0)	R (0)	0%
Ampicillin/sulbactam (20µg)	(≥15)	(≤1 3)	R (0)	R (0)	R (0)	0%
Cefepime (30µg)	(≥25)	(≤1 8)	R (0)	R (0)	R (0)	0%

Colistin (10µg)	(≥11)	(≤10)	R (0)	R (0)	R (0)	0%
Fosfomycin (10µg)	(≥16)	(≤12)	R (0)	R (0)	R (0)	0%
Meropenem (10µg)	(≥23)	(≤19)	I (22)	S (25)	S (26)	66%
MAR INDEX^f			0.92	0.92	0.83	

Table 3. Retention profile of phenolic and flavonoid compounds in SaO extract, SaO-Fe₃O₄NPs, and SaO-SeNPs as determined by HPLC.

Compound type	Compound name	Rt ^x (min)	SaO (µg/mL)	SaO-Fe ₃ O ₄ NPs (µg/mL)	SaO-SeNPs (µg/mL)
Phenoli	Chlorogenic	3.0	15.33	15.54	
	Catechol	4.0	13.54	10.21	6.33
	Syringenic	5.0	9.41	8.47	5.46
	Caffeic	8.0	22.74	17.36	10.19
Flavonoi	Pytrogallol	9.0	11.23	10.11	
	Ferulic	10.7	7.50	2.44	9.89
	Naringin	6.4	21.20		2.88
	Rutin	5.2	13.81		5.18
	Quersestin	7.0	12.75	16.70	13.36
	Kampferol	8.1	9.57	14.56	2.46
	Luteolin	9.0	11.36	5.17	5.26
	Apegenin	10.0	10.54	11.39	2.36
	Catechin	12.01	9.70	7.65	4.59

Rt^x: retention time (min)

Table 4. Antimicrobial activity of Fe₃O₄NPs and SeNPs against Gram-positive and Gram-negative bacteria using disc diffusion assay.

Metal nanoparticles	Tested bacteria	Type	Concentrations (µg/ml)						Mean		
			5	10	25	50	100	250			
			Inhibition zone (mm)								
Fe ₃ O ₄ NPs	<i>L. monocytogenes</i>	G ⁺	0.0±0.0	6.0±0.06	8.0±0.12	12.0±0.06	14.0±0.06	16.0±0.06	9.33±3.19		
			k	g	f	d	b	a	A		
			0.0±0.0	0.0±0.00	5.0±0.06	10.0±0.06	13.0±0.06	14.0±0.06	7.00±3.40		
	<i>B. cereus</i>	G ⁺	k	k	h	e	c	b	B		
			<i>Prot. mirabilis</i>	G ⁻	0.0±0.0	2.0±0.06	4.0±0.12	6.0±0.06	8.0±0.06	12.0±0.06	5.33±2.34
					k	j	i	g	f	d	C
	<i>P. aeruginosa</i>	G ⁻			0.0±0.0	0.0±0.0	5.0±0.10	6.0±0.12	10.0±0.06	14.0±0.06	5.83±3.00
			k	k	h	g	e	b	D		
			<i>E. coli</i>	G ⁻	0.0±0.0	0.0±0.0	2.0±0.10	8.0±0.06	10.0±0.12	12.0±0.06	5.33±2.88
	k	k			j	f	e	d	D		
	Mean	0.0±0.0			1.6±1.39	4.8±1.16	8.4±1.40	11.0±1.31	13.6±0.90		
			F	E	D	C	B	A			
SeNPs	<i>L. monocytogenes</i>	G ⁺	0.0±0.0	0.0±0.0	6.0±0.12	10.0±0.12	12.0±0.06	18.0±0.06	7.67±3.85		
			k	k	g	d	c	a	A		
			<i>B. cereus</i>	G ⁺	0.0±0.0	0.0±0.0	2.0±0.06	4.0±0.10	5.97±0.03	9.0±0.06	3.49±1.93
	k	k			j	i	g	e	D		
	<i>Prot. mirabilis</i>	G ⁻			0.0±0.0	4.0±0.06	6.0±0.12	8.0±0.06	12.0±0.06	14.0±0.06	7.33±2.80
			k	i	g	f	c	b	B		
			<i>P. aeruginosa</i>	G ⁻	0.0±0.0	0.0±0.0	5.0±0.10	10.0±0.12	12.0±0.06	14.0±0.06	6.83±3.30
	k	k			h	d	c	b	C		
	<i>E. coli</i>	G ⁻			0.0±0.0	0.0±0.0	2.0±0.06	4.0±0.06	6.0±0.06	8.0±0.12	3.33±1.77
			k	k	j	i	g	f	E		
			Mean	0.0±0.0	0.8±0.96	4.2±1.10	7.2±1.62	9.59±1.76	12.6±2.19		
			F	E	D	C	B	A			

Data are expressed as means ± standard error (SE). Means with different letters are significantly different according to the Least Significant Differences test (P < 0.01). The uppercase letters belong to the main effects of the studied factors (green for the tested bacteria and red for concentrations of MNPs), while the lowercase letters belong to the interaction effect between the two studied factors.

Table 4x. Antibacterial activity of Fe₃O₄NPs and SeNPs against the most sensitive Gram-positive bacterium (*Staphylococcus pasteurii*) using disc diffusion assay at lower concentrations.

Metal nanoparticles	Concentrations (µg /ml)						Mean
	0.25	0.5	1.0	5	10	25	
	Inhibition zone (mm)						
Fe ₃ O ₄ NPs	0.0±0.0 f	6.0±0.06 e	8.0±0.12 d	14.0±0.06 c	16.0±0.06 b	25.0±0.06 a	11.50±4.75
SeNPs	0.0±0.0 f	6.0±0.06 e	7.0±0.06 d	8.0±0.06 c	12.0±0.11 b	16.0±0.06 a	8.37±2.95

Data are expressed as means ± standard error (SE). Means with different letters are significantly different according to the Least Significant Differences test (P < 0.01). The lowercase letters belong to the interaction effect between the two studied factors.

Table 5. Antibacterial activity of SaO-Fe₃O₄NPs and SaO-SeNPs against Gram-positive and Gram-negative bacteria using disc diffusion assay.

Comp osite	Tested bacteria	Ty pe	Concentrations (µg/ml)							Mean
			0.25	0.5	1	5	10	25	50	
			Inhibition zone (mm)							
SaO- Fe ₃ O ₄ NPs	<i>L. monocytog enes</i>	G +	0.0± 0.0 m	0.0±0. 0 m	4.0±0. 06 j	6.0±0. 12 i	12.0±0 .06 e	14.0± 0.06 c	20.0± 0.06 a	8.00± 4.15 A
	<i>B. cereus</i>		0.0± 0.0 m	0.0±0. 0 m	2.0±0. 06 l	3.0±0. 06 k	8.0±0. 06 h	10.0± 0.06 q	12.0± 0.06 e	5.00± 2.70 D
										6.43± 3.19 C
										6.57± 4.31 B
	<i>E. coli</i>		0.0± 0.0 m	0.0±0. 0 m	0.0±0. 0 m	4.0±0. 06 j	8.0±0. 06 h	10.0± 0.06 g	12.0± 0.12 e	4.86± 2.82 D
	Mean		0.0± 0.0 F	0.0±0 .0 F	2.0±1 .07E	4.4±0 .80 D	9.2±0. 96 C	11.6± 0.97	16.0± 5.14A	

^a (The Clinical & Laboratory Standards Institute)

^b (EM100 connect - CLSI M100 ED30, 2024)

^c S, I & R (sensitive, intermediate & resistance)

^d AE Antibiotic effectiveness % = (No of Sensitive strain/total No of strain)

^f Multiple antibiotic resistance (MAR) index = a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

			B							
SaO-SeNPs	L. monocytogenes	G +	0.0±	4.0±0.	9.0±0.	12.0±	14.0±0	16.0±	20.0±	10.71 ±3.80
			0.0 r	06 o	06 j	0.12 g	.06 e	0.06 c	0.06 a	A
	B. cereus		0.0±	2.0±0.	4.0±0.	5.0±0.	6.0±0.	10.0±	14.0±	5.86±
			0.0 r	06 q	06 o	06 n	06 m	0.06 i	0.06 e	2.62 D
	Prot. mirabilis	G-	0.0±	4.0±0.	6.0±0.	7.0±0.	9.0±0.	12.0±	18.0±	8.00±
			0.0 r	06 o	06 m	12 l	06 j	0.06 g	0.06 b	3.18 B
	P. aeruginosa		0.0±	0.0±0.	2.0±0.	3.0±0.	10.0±0	13.0±	15.0±	6.14±
			0.0 r	0 r	06 q	06 p	.06 i	0.06 f	0.06 d	3.48 C
	E. coli		0.0±	2.0±0.	3.0±0.	4.0±0.	5.0±0.	8.0±0.	11.0±	4.71±
			0.0 r	06 q	06 p	06 o	06 n	06 k	0.12 h	2.04 E
Mean			0.0±	2.40±	4.80±	6.20±	8.80±	11.8±	15.6±	
			0.0 G	0.90 F	1.48 E	1.91 D	1.91 C	1.62 B	1.88 A	

Table 5x. Antibacterial activity of SaO-Fe₃O₄NPs and SaO-SeNPs against the most sensitive Gram-positive bacterium (*Staphylococcus pasteurii*) using disc diffusion assay at very low concentrations.

Composite	Concentrations (µg /ml)						Mean
	0.015	0.03	0.0625	0.125	0.25	0.5	
	Inhibition zone (mm)						
SaO-Fe₃O₄NPs	0.00±0.0 0 f	6.00±0.0 6 e	7.00±0.1 2 d	11.00±0.0 6 c	6.00±0.0 6 b	8.00±0.05 8 a	9.67±3.64
SaO-SeNPs	0.0±0.0 f	4.0±0.06 e	8.0±0.12 d	11.0±0.06 c	14.0±0.0 6 b	15.0±0.06 a	8.67±3.18

Data are expressed as means ± standard error (SE). Means with different letters are significantly different according to the Least Significant Differences test (P < 0.01). The lowercase letters belong to the interaction effect between the two studied factors.

Data are expressed as means ± standard error (SE). Means with different letters are significantly different according to the Least Significant Differences test (P < 0.01). The uppercase letters belong to the main effects of studied factors (green for the tested bacteria and red for the composites), while the lowercase letters belong to the

Table 6. Broth microdilution-based MIC values of the SaO extract, MNPs, and their composites against tested MDR bacteria, compared with meropenem.

Data are expressed as means $\pm$ standard error (SE). Means with different letters are significantly different according to the Least Significant Differences test ($P < 0.01$).

^a *Salvia officinalis* L. extract ^b Magnetite nanoparticles

Type	Tested bacteria	Minimum inhibitory concentration (MIC; $\mu\text{g/mL}$)					
		SaO ^a	Fe ₃ O ₄ NP ^b	SeNPs ^c	SaO-Fe ₃ O ₄ NP ^d	SaO-SeNPs ^f	Mero - penem
G	<i>S. pasteurii</i>	125 $\pm$ 0.11 5 c	0.50 $\pm$ 0.05 8 i	0.50 $\pm$ 0.11 5 i	0.03 $\pm$ 0.00 6 j	0.03 $\pm$ 0.00 6 j	0.25 $\pm$ 0.00 6 j
	<i>L. monocytogenes</i>	250 $\pm$ 0.10 0 b	10.0 $\pm$ 0.11 5 e	25.0 $\pm$ 0.05 8 d	1.00 $\pm$ 0.05 8 h	0.50 $\pm$ 0.05 8 i	0.50 $\pm$ 0.05 8 i
	<i>B. cereus</i>	250 $\pm$ 0.11 5 b	25.0 $\pm$ 0.05 8 d	25.0 $\pm$ 0.10 0 d	1.00 $\pm$ 0.10 0 h	0.50 $\pm$ 0.05 8 i	0.50 $\pm$ 0.05 8 i
C	<i>Prot. mirabilis</i>	500 $\pm$ 0.11 5 a	10.0 $\pm$ 0.05 8 e	10.0 $\pm$ 0.10 0 e	1.00 $\pm$ 0.10 0 h	0.50 $\pm$ 0.05 8 i	1.00 $\pm$ 0.10 0 h
	<i>p. aeruginosa</i>	250 $\pm$ 0.11 5 b	25.0 $\pm$ 0.11 5 d	25.0 $\pm$ 0.10 0 d	5.00 $\pm$ 0.05 8 f	1.00 $\pm$ 0.05 8 h	2.00 $\pm$ 0.05 8 g
	<i>E. coli</i>	500 $\pm$ 0.05 8 a	25.0 $\pm$ 0.10 0 d	25.0 $\pm$ 0.05 8 d	5.00 $\pm$ 0.10 0 f	0.50 $\pm$ 0.05 8 f	2.00 $\pm$ 0.10 0 g

^c Selenium nanoparticles
Salvia officinalis L. extract

^d Magnetite nanoparticles conjugated with

^f Selenium nanoparticles conjugated with *Salvia officinalis* L. extract

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Table 1. Antibiotic sensitivity of Gram-positive bacteria.

Antibiotics	Category & inhibition zone (mm) ^b		Classification of Tested bacteria (S-R-I) according to the inhibition zone (mm) ^c			% Antibiotic effectiveness (AE) ^d
	S	R	<i>S. pasteuri</i>	<i>B. cereus</i>	<i>L. monocytogenes</i>	
Azithromycin (15µg)	(≥18)	(≤13)	S (25)	R (6)	R (8)	33%
Chloramphenicol (30µg)	(≥18)	(≤12)	S (25)	I (15)	R (14)	33%
Doxycycline (30µg)	(≥19)	(≤14)	S (27)	R (14)	R (12)	33%
Gentamicin (10µg)	(≥15)	(≤12)	R (10)	R (12)	R (14)	0%
Levofloxacin (5µg)	(≥21)	(≤15)	R (20)	R (20)	I (18)	0%
Nitrofurantoin (300µg)	(≥17)	(≤14)	R (12)	R (10)	R (14)	0%
Trimethoprim (5µg)	(≥16)	(≤10)	R (10)	R (0)	R (0)	0%
Clindamycin (2µg)	(≥21)	(≤14)	R (12)	R (14)	I (18)	0%
Linezolid (30µg)	(≥21)	(≤20)	R (18)	R (16)	R (10)	0%
Oxacillin (30µg)	(≥22)	(≤21)	R (0)	R (0)	R (0)	0%
Rifampin (5µg)	(≥20)	(≤16)	R (0)	R (0)	R (0)	0%
Vancomycin (30µg)	(≥12)	(≤18)	R (0)	I (14)	R (5)	0%
Meropenem (10µg)	(≥23)	(≤19)	S (30)	S (27)	S (28)	100%
MAR INDEX	0.69 0.77 0.77					

a (The Clinical & Laboratory Standards Institute).

b (EM100 connect - CLSI M100 ED30, 2024).

c S, I & R (sensitive, intermediate & resistance).

d AE Antibiotic effectiveness % = (No of Sensitive strain/total No of strain).

f Multiple antibiotic resistance (MAR) index = a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

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Table 4. Surface charge characterization via zeta potential analysis

Sample	ζ -Potential (mV)	Std. Dev. (mV)	Stability Class	pH	Conductivity (mS/cm)
SeNPs	-28.5	± 2.1	Good	7.1	0.134
SaO-SeNPs	-35.2	± 2.4	Excellent	6.9	0.122
Fe ₃ O ₄ NPs	-21.3	± 1.8	Good	6.9	0.109
SaO-Fe ₃ O ₄ NPs	-32.8	± 2.6	Excellent	7.1	0.109
SaO Extract	-18.7	± 1.5	Moderate	6.8	0.096

Data are expressed as the standard deviation of triplicate independent measurements. Stability classification based on conventional criteria: Excellent ($|\zeta| > 30$ mV), Good ($20 < |\zeta| < 30$ mV), Moderate ($10 < |\zeta| < 20$ mV). Measurements performed via electrophoretic light scattering at 25°C using the Malvern Zetasizer system. The enhanced negative surface charge in bio-conjugated nanocomposites confirms successful phytochemical adsorption and provides electrostatic stabilization, preventing particle aggregation.

Table 5. Retention profile of phenolic and flavonoid compounds in SaO extract, SaO-Fe₃O₄NPs, and SaO-SeNPs as determined by HPLC.

Compound type	Compound name	Rt ^x (min)	SaO (µg/mL)	SaO-Fe ₃ O ₄ NPs (µg/mL)	SaO-SeNPs (µg/mL)
Phenol	Chlorogenic	3.0	15.33	15.54	
	Catechol	4.0	13.54	10.21	6.33
	Syringenic	5.0	9.41	8.47	5.46
	Caffeic	8.0	22.74	17.36	10.19
	Pyrogallol	9.0	11.23	10.11	
	Ferulic	10.7	7.50	2.44	9.89
Flavonoi	Naringin	6.4	21.20		2.88
	Rutin	5.2	13.81		5.18
	Quersestin	7.0	12.75	16.70	13.36
	Kampferol	8.1	9.57	14.56	2.46
	Luteolin	9.0	11.36	5.17	5.26
	Apegenin	10.0	10.54	11.39	2.36
	Catechin	12.01	9.70	7.65	4.59

Rt^x: retention time (min)

Table 6. Antimicrobial activity of Fe₃O₄NPs and SeNPs against Gram-positive and Gram-negative bacteria using disc diffusion assay.

Metal nanoparticles	Tested bacteria	Type	Concentrations (µg/ml)						Mean
			5	10	25	50	100	250	
Inhibition zone (mm)									
Fe ₃ O ₄ NPs	<i>L. monocytogenes</i>	G+	0.0±0.0 _{0 k}	6.0±0.06 _g	8.0±0.12 _f	12.0±0.0 _{6 d}	14.0±0.0 _{6 b}	16.0±0.0 _{6 a}	9.33±3.1 _{9 A}
	<i>B. cereus</i>		0.0±0.0 _{0 k}	0.0±0.00 _k	5.0±0.06 _h	10.0±0.0 _{6 e}	13.0±0.0 _{6 c}	14.0±0.0 _{6 b}	7.00±3.4 _{0 B}
	<i>P. mirabilis</i>	G-	0.0±0.0 _{0 k}	2.0±0.06 _j	4.0±0.12 _i	6.0±0.06 _g	8.0±0.06 _f	12.0±0.0 _{6 d}	5.33±2.3 _{4 C}
	<i>P. aeruginosa</i>		0.0±0.0 _{0 k}	0.0±0.0 _k	5.0±0.10 _h	6.0±0.12 _g	10.0±0.0 _{6 e}	14.0±0.0 _{6 b}	5.83±3.0 _{0 D}
	<i>E. coli</i>		0.0±0.0 _{0 k}	0.0±0.0 _k	2.0±0.10 _j	8.0±0.06 _f	10.0±0.1 _{2 e}	12.0±0.0 _{6 d}	5.33±2.8 _{8 D}
	Mean		0.0±0.0 _{0 F}	1.6±1.3 _{9 E}	4.8±1.1 _{6 D}	8.4±1.40 _C	11.0±1.3 _{1 B}	13.6±0.9 _{0 A}	
SeNPs	<i>L. monocytogenes</i>	G+	0.0±0.0 _{0 k}	0.0±0.0 _k	6.0±0.12 _g	10.0±0.1 _{2 d}	12.0±0.06 _c	18.0±0.0 _{6 a}	7.67±3.8 _{5 A}
	<i>B. cereus</i>		0.0±0.0 _{0 k}	0.0±0.0 _k	2.0±0.06 _j	4.0±0.10 _i	5.97±0.03 _g	9.0±0.06 _e	3.49±1.9 _{3 D}
	<i>P. mirabilis</i>	G-	0.0±0.0 _{0 k}	4.0±0.06 _i	6.0±0.12 _g	8.0±0.06 _f	12.0±0.06 _c	14.0±0.0 _{6 b}	7.33±2.8 _{0 B}
	<i>P. aeruginosa</i>		0.0±0.0 _{0 k}	0.0±0.0 _k	5.0±0.10 _h	10.0±0.1 _{2 d}	12.0±0.06 _c	14.0±0.0 _{6 b}	6.83±3.3 _{0 C}
	<i>E. coli</i>		0.0±0.0 _{0 k}	0.0±0.0 _k	2.0±0.06 _j	4.0±0.06 _i	6.0±0.06 _g	8.0±0.12 _f	3.33±1.7 _{7 E}
	Mean		0.0±0.0 _{0 F}	0.8±0.9 _{6 E}	4.2±1.1 _{0 D}	7.2±1.62 _C	9.59±1.7 _{6 B}	12.6±2.1 _{9 A}	

Data are expressed as means ± standard error (SE). Means with different superscript letters are significantly different according to the Least Significant Differences test (P < 0.01). The uppercase letters belong to the main

effects of the studied factors (green for the tested bacteria and red for concentrations of MNPs), while the lowercase letters belong to the interaction effect between the two studied factors.

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Table 6x. Antibacterial activity of Fe₃O₄NPs and SeNPs against the most sensitive Gram-positive bacterium (*Staphylococcus pasteurii*) using disc diffusion assay at lower concentrations.

Metal nanoparticles	Concentrations (µg /ml)						Mean
	0.25	0.5	1.0	5	10	25	
	Inhibition zone (mm)						
Fe₃O₄NPs	0.0±0.0 f	6.0±0.0 6 e	8.0±0.12 d	14.0±0.0 6 c	16.0±0.0 6 b	25.0±0.0 6 a	11.50±4.75
SeNPs	0.0±0.0 f	6.0±0.0 6 e	7.0±0.06 d	8.0±0.06 c	12.0±0.1 1 b	16.0±0.0 6 a	8.37±2.95

Data are expressed as means ± standard error (SE). Different superscript letters are significantly different according to the Least Significant Differences test ($P < 0.01$). The lowercase letters belong to the interaction effect between the two studied factors.

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Table 3. Hydrodynamic diameter characterization by dynamic light scattering (DLS) analysis

Sample	Mean Size (nm)	Std. Dev. (nm)	PDI	Temp. (°C)	Medium
SeNPs	69.4	± 3.2	0.185	25.0	Aqueous
SaO-SeNPs	69.4	± 3.8	0.238	25.0	Aqueous
Fe ₃ O ₄ NPs	74.3	± 4.5	0.231	25.0	Aqueous
SaO-Fe ₃ O ₄ NPs	74.3	± 4.2	0.155	25.0	Aqueous
SaO Extract	31.5	± 2.1	0.209	25.0	Aqueous

Data are expressed as the standard deviation of triplicate independent measurements. PDI: Polydispersity Index, a dimensionless measure of size distribution breadth (values < 0.3 indicate acceptable monodispersity for pharmaceutical applications). All measurements were performed using the Malvern Zetasizer system with 173° backscatter detection angle.

Table 7x. Antibacterial activity of SaO-Fe₃O₄NPs and SaO-SeNPs against the most sensitive Gram-positive bacterium (*Staphylococcus pasteurii*) using disc diffusion assay at very low concentrations.

Composite	Concentrations (µg /ml)						Mean
	0.015	0.03	0.0625	0.125	0.25	0.5	
	Inhibition zone (mm)						
SaO-Fe₃O₄NPs	0.00±0.0 0 ^f	6.00±0.0 6 ^e	7.00±0.1 2 ^d	11.00±0.0 6 ^c	6.00±0.0 6 ^b	8.00±0.05 8 ^a	9.67±3.64
SaO-SeNPs	0.0±0.0 ^f	4.0±0.06 e	8.0±0.12 d	11.0±0.06 c	14.0±0.0 6 ^b	15.0±0.06 a	8.67±3.18

Data are expressed as means ± standard error (SE). Different superscript letters are significantly different according to the Least Significant Differences test (P < 0.01). The lowercase letters belong to the interaction effect between the two studied factors.

Table 7. Antibacterial activity of SaO-Fe₃O₄NPs and SaO-SeNPs against Gram-positive and Gram-negative bacteria using disc diffusion assay.

Compos ite	Tested bacteria	Type	Concentrations (µg/ml)							Mean
			0.25	0.5	1	5	10	25	50	
			Inhibition zone (mm)							
SaO- Fe ₃ O ₄ N Ps	<i>L. monocytogenes</i>	G+	0.0±0.0 _m	0.0±0.0 _m	4.0±0.06 _j	6.0±0.12 _i	12.0±0.06 _e	14.0±0.0 _{6 c}	20.0±0.06 _a	8.00±4.15 _A
	<i>B. cereus</i>		0.0±0.0 _m	0.0±0.0 _m	2.0±0.06 _l	3.0±0.06 _k	8.0±0.06 _h	10.0±0.0 _{6 g}	12.0±0.06 _e	5.00±2.70 _D
	<i>P. mirabilis</i>	G-	0.0±0.0 _m	0.0±0.0 _m	4.0±0.06 _j	6.0±0.12 _i	8.0±0.06 _h	11.0±0.0 _{6 f}	16.0±0.06 _b	6.43±3.19 _C
	<i>P. aeruginosa</i>		0.0±0.0 _m	0.0±0.0 _m	0.0±0.0 _m	3.0±0.06 _k	10.0±0.06 _g	13.0±0.0 _{6 d}	20.0±0.06 _a	6.57±4.31 _B
	<i>E. coli</i>		0.0±0.0 _m	0.0±0.0 _m	0.0±0.0 _m	4.0±0.06 _j	8.0±0.06 _h	10.0±0.0 _{6 g}	12.0±0.12 _e	4.86±2.82 _D
	Mean		0.0±0.0 _F	0.0±0.0 _F	2.0±1.07 _F	4.4±0.80 _D	9.2±0.96 _C	11.6±0.97 _B	16.0±5.14 _A	
SaO- SeNPs	<i>L. monocytogenes</i>	G+	0.0±0.0 _r	4.0±0.06 _o	9.0±0.06 _j	12.0±0.12 _g	14.0±0.06 _e	16.0±0.0 _{6 c}	20.0±0.06 _a	10.71±3.80 _A
	<i>B. cereus</i>		0.0±0.0 _r	2.0±0.06 _q	4.0±0.06 _o	5.0±0.06 _n	6.0±0.06 _m	10.0±0.0 _{6 i}	14.0±0.06 _e	5.86±2.62 _D
	<i>P. mirabilis</i>	G-	0.0±0.0 _r	4.0±0.06 _o	6.0±0.06 _m	7.0±0.12 _i	9.0±0.06 _j	12.0±0.0 _{6 g}	18.0±0.06 _b	8.00±3.18 _B
	<i>P. aeruginosa</i>		0.0±0.0 _r	0.0±0.0 _r	2.0±0.06 _q	3.0±0.06 _p	10.0±0.06 _i	13.0±0.0 _{6 f}	15.0±0.06 _d	6.14±3.48 _C
	<i>E. coli</i>		0.0±0.0 _r	2.0±0.06 _q	3.0±0.06 _p	4.0±0.06 _o	5.0±0.06 _n	8.0±0.06 _k	11.0±0.12 _h	4.71±2.04 _E
	Mean		0.0±0.0 _G	2.40±0.90 _F	4.80±1.40 _F	6.20±1.91 _D	8.80±1.91 _C	11.8±1.62 _B	15.6±1.80 _A	

Data are expressed as means ± standard error (SE). Means with different superscript letters are significantly different according to the Least Significant Differences test (P < 0.01). The uppercase letters belong to the main effects of studied factors (green for the tested bacteria and red for concentration of the composites), while the lowercase letters belong to the interaction effect between the two studied factors.

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Table 8. Broth microdilution-based MIC values of the SaO extract, MNPs, and their composites against tested MDR bacteria, compared with meropenem.

Type	Tested bacteria	Minimum inhibitory concentration (MIC; µg/mL)					
		SaO	Fe ₃ O ₄ NPs	SeNPs	SaO-Fe ₃ O ₄ NPs	SaO-SeNPs	Mero - penem
G	<i>S. pasteurii</i>	125±0.11 5 ^c	0.50±0.05 8 ⁱ	0.50±0.11 5 ⁱ	0.03±0.00 6 ^j	0.03±0.00 6 ^j	0.25±0.00 6 ^j
	<i>L. monocytogenes</i>	250±0.10 0 ^b	10.0±0.11 5 ^e	25.0±0.05 8 ^d	1.00±0.05 8 ^h	0.50±0.05 8 ⁱ	0.50±0.05 8 ⁱ
	<i>B. cereus</i>	250±0.11 5 ^b	25.0±0.05 8 ^d	25.0±0.10 0 ^d	1.00±0.10 0 ^h	0.50±0.05 8 ⁱ	0.50±0.05 8 ⁱ
C	<i>P. mirabilis</i>	500±0.11 5 ^a	10.0±0.05 8 ^e	10.0±0.10 0 ^e	1.00±0.10 0 ^h	0.50±0.05 8 ⁱ	1.00±0.10 0 ^h
	<i>p. aeruginosa</i>	250±0.11 5 ^b	25.0±0.11 5 ^d	25.0±0.10 0 ^d	5.00±0.05 8 ^f	1.00±0.05 8 ^h	2.00±0.05 8 ^g
	<i>E. coli</i>	500±0.05 8 ^a	25.0±0.10 0 ^d	25.0±0.05 8 ^d	5.00±0.10 0 ^f	0.50±0.05 8 ^f	2.00±0.10 0 ^g

Data are expressed as means ± standard error (SE). Different superscript letters within the same row indicate significant differences among treatments according to the Least Significant Difference (LSD) test (P < 0.01).

Table 2. Antibiotic sensitivity of Gram-negative bacteria.

Antibiotics	Category & inhibition zone (mm) ^b		Classification of Tested bacteria (S-R-I) according to the Inhibition zone (mm) ^c			% Antibiotic effectiveness (AE) ^d
	S	R	<i>E. coli</i>	<i>P. mirabilis</i>	<i>P. aeruginosa</i>	
Azithromycin (15µg)	(≥18)	(≤13)	R (8)	R (8)	R (8)	0%
Chloramphenicol (30µg)	(≥18)	(≤12)	R (12)	R (12)	R (10)	0%
Doxycycline (30µg)	(≥19)	(≤14)	R (12)	R (13)	R (12)	0%
Gentamicin (10µg)	(≥15)	(≤12)	R (12)	R (8)	I (14)	0%
Levofloxacin (5µg)	(≥21)	(≤15)	R (15)	R (14)	R (6)	0%
Nitrofurantoin (300µg)	(≥17)	(≤14)	R (8)	R (0)	R (0)	0%
Trimethoprim (5µg)	(≥16)	(≤10)	R (0)	R (0)	R (0)	0%
Ampicillin/sulbactam (20µg)	(≥15)	(≤13)	R (0)	R (0)	R (0)	0%
Cefepime (30µg)	(≥25)	(≤18)	R (0)	R (0)	R (0)	0%
Colistin (10µg)	(≥11)	(≤10)	R (0)	R (0)	R (0)	0%
Fosfomycin (10µg)	(≥16)	(≤12)	R (0)	R (0)	R (0)	0%
Meropenem (10µg)	(≥23)	(≤19)	I (22)	S (25)	S (26)	66%

^a (The Clinical & Laboratory Standards Institute)

^b (EM100 connect - CLSI M100 ED30, 2024)

^c S, I & R (sensitive, intermediate & resistance)

^d AE Antibiotic effectiveness % = (No of Sensitive strain/total No of strain)

^f Multiple antibiotic resistance (MAR) index = a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.