

## Article

# Synergistic Effect of Magnetic Iron Oxide Nanoparticles with Medicinal Plant Extracts against Resistant Bacterial Strains

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**Abstract:** Nanoparticles are emerging as a fascinating alternative to antibiotics. When stabilized by chemical compounds, magnetite nanoparticles (MagNPs) consistently exhibit bactericidal effects across different types of bacteria. This study describes the synthesis, characterization, and antibacterial properties of magnetite MagNPs prepared by the coprecipitation method under continuous sonication. Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), and Dynamic Light Scattering (DLS) techniques revealed Fe<sub>3</sub>O<sub>4</sub>-NPs as spherical, uniform particles with an average size of approximately 16 nm. The antibacterial efficacy of MagNPs was investigated by combining them with methanolic extracts of three medicinal plants known for their antibacterial properties: *Aloysia triphylla*, *Sarcopoterium spinosum*, and *Urtica pilulifera*. The combined effect was assessed against both wild type and resistant strains of *Staphylococcus aureus* and *Escherichia coli*. The antibacterial synergistic effect of MagNPs and plant extracts was evaluated by the MIC test, which showed significant inhibitory properties against the growth of the four bacterial strains as compared to control samples of plant extracts alone. Furthermore, the synergistic effect of MagNPs combined with extracts from *Rosmarinus officinalis*, *Anchusa azurea*, *Quercus infectoria*, and *Urtica pilulifera* significantly prevented biofilm development in both sensitive and resistant strains of *Staphylococcus aureus*.

**Keywords:** magnetic nanoparticles; antibacterial; bacterial inhibition; *Escherichia*; *Staphylococcus*; Scanning Electron Microscopy; X-ray diffraction; Dynamic Light Scattering



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## 1. Introduction

Nanoparticles (NPs) are minute materials with diameters ranging from 1 to 100 nm, comparable in size to the molecular scale of living cells. They find extensive applications across various scientific sectors. The advent of nanoparticles has sparked a revolution in medicine with a wide variety of applications such as drug delivery, tissue repair, infection control, diagnostics, and bactericidal action [1–4]. The universal calamity of the ongoing emergence of antibiotic-resistant strains among patients poses a significant challenge, prompting researchers to seek an optimistic solution [5,6], with NPs being among the promising avenues [3,7,8]. To date, several types of metal nanoparticles have been explored for their antimicrobial effects, including silver nanoparticles (Ag-NPs), which are effective bactericides against *Escherichia coli* [9] and inhibitory for *Staphylococcus aureus* [10], albeit

concerns have been raised regarding their environmental toxicity [11]. Gold nanoparticles (Au-NPs) exhibit functional bactericidal effects against Gram-negative and Gram-positive bacteria, particularly when capped with cefaclor [12]. Combining Au-NPs with grapefruit extract has yielded promising results against *S. aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* [13]. Meanwhile, magnetic iron oxide NPs have been used for a long time in a variety of biomedical applications, including magnetic resonance imaging (MRI) contrast agents for cell identification, drug delivery systems, and cancer treatments [2,4]. Although magnetic iron oxide NPs were reported to have neurotoxicity in rats [14] other research has reported they are not cytotoxic in *in vivo* and *in vitro* assays [15]. These contradictory reports show that further research is required to resolve this controversial point [16]. The unique characteristics of iron oxide NPs make them more compatible for biomedical applications [17]. Magnetic iron oxide NPs are non-toxic to normal mammalian cells when conjugated with an antifungal drug [18] and enhance calcium precipitation in osteoblast cells, hence increasing bone density [19]. Furthermore, they offer advantages over other metal-NPs due to their cost-effectiveness, easy construction methodology, eco-friendliness, and the ability to be manipulated or removed from the treated tissue by an external magnetic field [4,20].

Despite these advantages, the antimicrobial effect of MagNPs has not been thoroughly investigated [21–25]. A study by Auffan et al. (2008) investigated three types of Iron-based NPs and their cytotoxicity toward *E. coli*. They found that maghemite  $\gamma\text{Fe}_2\text{O}_3$  had no observable toxicity toward *E. coli* due to their stable structure [21]. However, coating it with polyrhodanine amplified its effect not only against *E. coli* but also *S. aureus* [22]. In contrast, MagNPs and Zero valent Iron NPs (Fe-NPs) demonstrated potent bactericidal effects against *E. coli* by inducing oxidative stress inside cells [21]. Moreover, Kim (2010) reported that Fe-NPs exerted a stronger effect under anaerobic conditions, compared to aerobic conditions depending on the dose and duration of exposure [23]. This inactivation was attributed to the generation of intracellular oxidants, which compromised membrane integrity and respiratory activity [23]. Similarly, MagNPs stabilized by polyvinyl alcohol (PVA) exerted a toxic effect on *S. aureus* [24]. The interaction between MagNPs and bacterial interfaces was further explored by Arakha and colleagues (2015). They demonstrated that the negatively charged surface of MagNPs, when coated with positively charged chitosan, enhanced the bactericidal effect against both Gram-positive and Gram-negative bacteria [25]. Similarly, MagNPs coated with a shell of conjugated silicon-amine groups ( $\text{SiO}_2\text{-NH}_2$ ) effectively targeted multiple drug-resistant (MDR) *S. aureus* and *E. coli* [26], destroying their cells and preventing biofilm formation. In short, the hypothesis correlating the effect of stabilizing MagNPs with different materials and their ability to prevent oxidation requires further investigation.

Today, there is an increasing demand to return to ethnomedicine and natural plant products for several types of disease treatments, drug discoveries, and health-care issues. Biologically active compounds found in several plant species have been reported over the years to be effective drugs with minimal side effects in what is conventionally known as traditional medicine [27]. Enhancements in the effect of these biologically active compounds are reported when combined with metal NPs. An *in vitro* study by Arokiyaraj (2013) on  $\text{Fe}_3\text{O}_4$ -NPs treated with leaf extract of *Argemone mexicana* yielded significant antimicrobial results against *E. coli* and *Proteus mirabilis* [28]. A different study conducted by Shahriary et al. (2018) found that the combination of leaf extract (*Stachys lavandulifolia*) with a mixture of MagNPs/Ag-NPs exhibited good bactericidal effects against *E. coli* and *S. aureus* [29]. Previous findings have indicated that using MagNPs alone against the tested bacteria is not effective. However, Dinali et al. (2017) discussed the relevant reasons that may decrease their effectiveness, such as low particle stability, agglomeration, low bioavailability caused by undispersed particles, and an overall negative charge. Due to these reasons, using MagNPs requires conjugation or coating with other materials, which is feasible due to their nature [30,31].

On the other hand, the recurring crisis of antibiotic resistance among various pathogenic strains presents a formidable challenge for scientists, urging them to find optimistic so-

lutions without needing to resort to drastic measures as a final option. Using various compounds as alternatives for antibiotics is not enough to solve the challenge of resistance because bacterial strains overcome them with the passage of time. Three innovative solutions have emerged to overcome this problem: leveraging plant secondary metabolites [32], nanoparticles [7], or combinations thereof [33,34]. The strategy of combining different agents has shown promise in most cases, including the pairing of two or more antibiotics, antibiotics with plant extracts, antibiotics with non-antibacterial agents, and antibiotics with NPs [32,35–37].

Recent findings confirm that mixing weak bactericidal agents has a synergistic effect against multidrug-resistant (MDR) bacterial strains [33,38,39]. According to our knowledge, this study represents the first instance where chemically synthesized magnetite nanoparticles are combined with methanolic extracts of folkloric plants to evaluate their antibacterial efficacy. This innovative combination, integrating chemically synthesized magnetite NPs and methanolic extracts from diverse plants with known antibacterial properties, is anticipated to significantly enhance the antibacterial efficacy of both components against American Type Culture Collection strains (ATCC).

## 2. Materials and Methods

The chemicals used in this research were of reagent grade and used as received from the suppliers. This included ferrous and ferric chloride salts (Riedel-de Haën AG, Seelze, Germany); ammonium hydroxide (25%, Thermo Fisher Scientific, Hampton, NH, USA); absolute ethanol (AR grade, VWR chemicals, Radnor, PA, USA); absolute methanol (IsoLab Chemicals, Wertheim, Germany); Müller Hinton Agar (MHA) and broth (Oxoid Limited, Basingstoke, UK | Thermo Fisher Scientific—USA); iodinitrotetrazolium chloride (INT, 98%, Acros Organics | Thermo Fisher Scientific, Hampton, NH, USA); tryptone soya agar (TSA); Luria Britani agar (LBA), and broth (Biolab Diagnostics Laboratory Inc., Budapest, Hungary); glucose (anhydrous molecular biology grade, GeneON GmbH, Ludwigshafen, Germany); normal saline (NS, 0.9% *w/v* sodium chloride, pharmaceutical solutions industry, Jaddah, Saudi Arabia); and phosphate-buffered saline (PBS, 10X, HiMedia Laboratories, Pvt. Ltd., Mumbai, India). Ultrasonication was performed using ULTRASONIC LC 20H (Elma Ultrasonic Cleaners, Singen am Hohentwiel, Germany). The SEM images were obtained from the Quanta FEG 450 (FEI company, Hillsboro, OR, USA). The XRD data were obtained on Ultima IV X-Ray diffractometer, Rigaku, Japan (40 kV/40 mA, 3 degree/min, 15–90-degree, Cu K $\alpha$  radiation ( $\lambda = 1.548 \text{ \AA}$ ). The particle size was calculated using Scherrer formula. The grain pattern was compared to the ideal pattern of pure magnetite. The hydrodynamic size and surface charge of NPs were examined by Dynamic Light Scattering (DLS). A 1 mg/mL aqueous suspension of the NPs was prepared in distilled water and sonicated for 10 min. One hundred  $\mu\text{L}$  of the sonicated solution was then diluted 100 folds in distilled water and sonicated for 10 min. Finally, 2 mL of the sonicated solution was evaluated in the Malvern Zetasizer Nano ZS 90 device for size and zeta potential (Malvern Panalytical Ltd., Malvern, UK). Equipment used included an orbital shaker SSL1 from Stuart, Cole-Parmer Ltd., St Neots, UK, a vacuum rotary evaporator HAHNVAPOR from Hahnshin Scientific, Gimpo, Republic of Korea, a grinder from IKA Mill M20 Universal mill (IKA-Werke GmbH & Co. KG Staufen, Germany), a spectrophotometer (BioMate3, Thermo spectronic Thermo Fisher Scientific, Waltham, MA, USA) And a microplate reader from BioTek, Synergy2 SL Luminescence Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA.

### 2.1. Synthesis of MagNPs

The MagNPs were synthesized using the coprecipitation method by mixing  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  (1.99 g) and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (5.41 g) in 2:1 molar ratio in 100 mL distilled water under continuous sonication (50–65 °C, 20–40 min).  $\text{NH}_3$  (25%, 8 mL) were added dropwise at intervals of 30–40 min where a black precipitation appeared immediately. Sonication continued for about 1 h. and was left to settle overnight at room temperature. The NPs precipitate was separated by filtration using Whatman filter paper no. 1001-150 [40], washed with plenty of

deionized water and absolute ethanol three times, and dried in an oven (60–80 °C, 1–2 days). This process produced about 2.31 g of dry black NPs, which were collected and ground into fine powder using a mortar and pestle and kept in a contained container for further analysis, characterization, and use. All equipment, including containers, Whatman filter papers, and solutions, was sterilized using an autoclave and used under sterile conditions. The prepared MagNPs were structurally characterized by Scanning Electron Microscopy (SEM), X-ray diffraction (XRD), and Dynamic Light Scattering (DLS) [17,41–43].

## 2.2. Plant Material Collection

Seven traditional medicinal plant species were utilized to assess the impact of their extracts when combined with Fe<sub>3</sub>O<sub>4</sub>-NPs on bacterial growth or inhibition (Table A1). Among these, four species were wild plant species, namely, *Urtica pilulifera* L., *Anchusa azurea* Mill., *Sarcopoterium spinosum* (L.) Spach, *Quercus infectoria* Olivier, while the other two species, *Rosmarinus officinalis* L. and *Aloysia triphylla* (L'Hér.) Britton, are cultivated species. The materials were collected from their natural habitat in northern Jordan during the period of March to May 2021 for wild species and from the JUST campus for cultivated species. The collected plant materials have been authenticated by Dr. Mohammad Al-Gharaibeh (Department of Plant Production, Jordan University of Science and Technology).

## 2.3. Methanolic Extract Preparation

The plants were collected, dried, and grounded at room temperature in the shade. The selected plant parts for use were processed in a grinder with 100 g of the resulting ground material mixed with 500 mL of methanol and left to sit for 72 h on an orbital shaker at room temperature. The resulting dried extract was then stored in a refrigerator until needed, following the procedure outlined in the literature [44]. The plant list is found in Table A1.

## 2.4. Bacterial Strains

Four bacterial strains obtained from the American Type Culture Collection (ATCC) were used in the antibacterial assays, which are *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 43300, *Escherichia coli* ATCC 25922, and *E. coli* BAA 2452. Bacterial stocks were preserved as aliquots in Mueller Hinton Broth (MHB) with 10% glycerol at −80 °C.

## 2.5. Antibacterial Assay of Combined MagNPs—Plant Extracts

Different analytical methods were used to evaluate the bactericidal effect of the combined MagNPs-plant extract, including Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), and biofilm formation assay. Antibacterial assays were performed for each plant extract alone and then for the combination of plant extract and MagNPs together after mixing 2MIC of the plant extract and 128 mg/mL of MagNPs for 18–24 h [28,45]. Fresh cultures from cryogenic samples were cultured on MHA incubated overnight at 37 °C, then, one colony was picked into 2 mL MHB incubated for 2 h to prepare 0.5 McFarland standard at 600 nm and optical density (OD) (0.08–0.13) using a spectrophotometer. Biofilm experiment preparation was performed by culturing cryopreserved samples on LBA for about 20 h, then one colony was picked up and suspended in 10 mL TSB, supplemented with 1% glucose, incubated at 37 °C overnight. The OD was measured at 600 nm, then diluted with TSB supplemented with 1% glucose to get 0.1 OD suspension.

## 2.6. The Minimal Inhibitory Concentration (MIC)

A minimal inhibitory concentration is defined as the least concentration of an agent that inhibits the growth of the bacteria and is observed by the visible eye. Each plant extract (128 mg/mL) was prepared in MHB according to Clinical and Laboratory Standards Institute (CLSI) guidelines [46], and 100 µL was put in the first well of a 96 well plate, 2 fold serial dilutions were performed in 9 wells and the last 50 µL was discarded. Fifty µL of 0.5 McFarland standard inoculum were prepared, then diluted 1:10 in MHB and added

to each well. The growth control consisted of inoculum in MHB, and the sterility control consisted of MHB only [44,47]. The plates were incubated at 37 °C for 16–20 h. Triplicate and replicate plates were run together for each strain and each agent. A reference plate was prepared from the plant extract using a serial dilution similar to the test plates. However, MHB was added instead of culture to assess for contamination in the plant extracts. After the incubation period, 40 µL of a 0.2 mg/mL solution of Iodonitrotetrazolium chloride (INT) indicator were added to each well after being sterilized with a Mini pore syringe filter (22 µm), and the samples were incubated for 30–40 min at 37 °C. Any color change to red or dark red indicated that there was growth in the well. The lowest concentration of the agent that did not cause a color change was considered the MIC. The addition of INT took place within the reported incubation time of the MIC assay. A positive control of chloramphenicol antibiotic was also included in the MIC assay to ensure that the assay was working properly.

### 2.7. Biofilm Formation Assay

To determine if the strains used in this study are biofilm formers, using a 96 well plate, 200 µL of each strain was inoculated in TSB (supplemented with 1% glucose [48] with OD 0.1 at 600 nm. In addition, *S. aureus* ATCC 33591, which is a strong biofilm former, was used for comparison [49]. Then, the plates were incubated at 37 °C for 24 h. The plate contents were discarded directly and washed with PBS 200 µL three times. Plates were allowed to dry at room temperature for 20 min, then placed in an oven at 60 °C for heat fixation. Crystal violet 0.1% solution 170 µL was added to stain the formed biofilm for 20 min at room temperature. Then, the dye was discarded after the incubation time, and the plates were washed with plenty of tap water until a clear solution appeared. The plates were left to dry at room temperature for 2 h at least (or overnight). Next, 200 µL of 80% Ethanol and 20% Acetone solution was added, incubated for 30 min at room temperature, and then the results were taken using a plate reader at 570 nm. The experiment was performed in triplicate and replicated on the same day. The strength of the biofilm was determined according to the average OD of the sterility control (SC) and the standard deviation (SD) of the readings, as reported in Singh et al. (2017) [49]. The SC was TSB alone. The biofilm strength was calculated according to the mentioned equation: OD Cutoff value = average OD<sub>negative control</sub> + 3 × SD of OD<sub>negative control</sub>. If the OD average of the strain ≤ OD cutoff value, the strain is a non-biofilm former (N). If OD cutoff value < OD average of the strain ≤ 2 × OD cutoff value, the strain is a weak biofilm former (W). If 2 × OD cutoff value < OD average of the strain ≤ 4 × OD cutoff value, the strain is a moderate biofilm former (M). And if 4 × OD cutoff value < OD average of the strain, the strain is a strong biofilm former.

### 2.8. Anti-Biofilm Formation Assay

After determining the strains' ability to form biofilms, an assay was used to evaluate the effect of plant extract combined with MagNPs to prevent biofilm development in moderate and strong biofilm formers. In microtiter plate 100 µL of inoculum with OD 0.1 at 600 nm was added and incubated for 30 min to allow bacterial cell attachment. Different concentrations of the agents were added, including 2×MIC, MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC of the plant extract alone, MagNPs alone or combination of both (16–20 h combined). Plates were incubated for 24 h at 37 °C. The SC were prepared from TSB alone supplemented with 1% glucose, the GC were prepared from inoculum with TSB supplemented with 1% glucose, reference wells were prepared from the different concentrations of agent with TSB alone, supplemented with 1% glucose, to eliminate color effects from plate reading.

### 2.9. Statistical Analysis

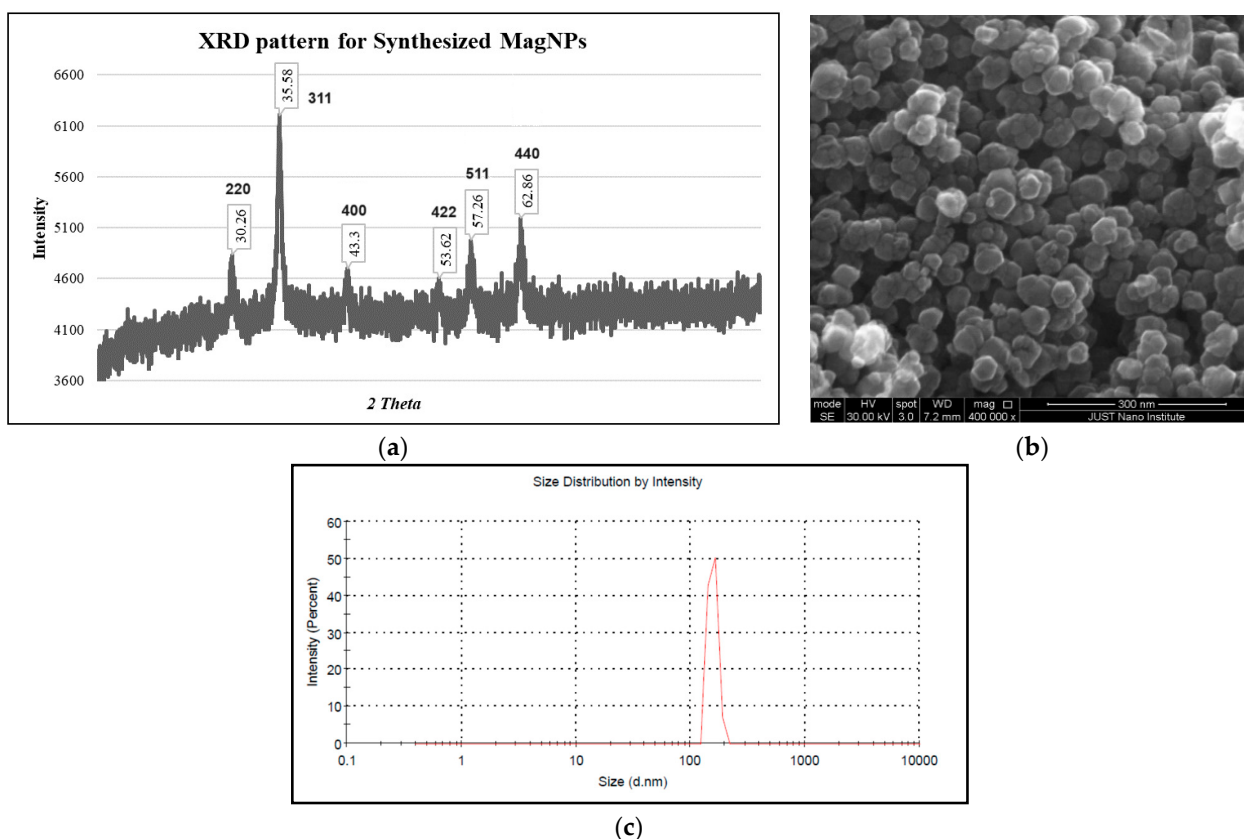
The values of MIC were reported as the means ± SD of 9 results. JMP program version 17.0 from SAS Institute Inc. (Cary, NC, USA, 2022) was used to analyze the data. The data were not normally distributed, as assessed using the Q-Q plot test. Therefore, the nonparametric Kruskal-Wallis test and Wilcoxon rank test were used to compare the MIC

values of plant extract alone and the MIC of plant extract combined with MagNPs. In addition, the Wilcoxon rank test was used to contrast the MIC values of MagNPs alone and MagNPs combined with plant extract against *S. aureus* strains. The nonparametric Kruskal-Wallis test was used to compare the percentage of biofilm inhibition after treating *S. aureus* strains with plant extract alone and plant extract combined with MagNPs. A  $p$ -value  $\leq 0.01$  was considered significant.

### 3. Results

The structure of the obtained powder was examined by three techniques, namely, XRD, SEM, and DLS. The powder-XRD in Figure 1a showed 6 diffraction peaks at  $2\theta$  of  $30.26^\circ$ ,  $35.58^\circ$ ,  $43.30^\circ$ ,  $53.62^\circ$ ,  $57.26^\circ$ ,  $62.86^\circ$  degrees due to the Bragg's Plane of 220, 311, 400, 422, 511, and 440 with the inverse cubic spinel structure of  $\text{Fe}_3\text{O}_4$  corresponding to Fd-3m space group [50] in agreement with the standard XRD pattern of magnetite NPs card # 01-071-6336 in the literature database of  $2\theta$  values of  $30.24^\circ$ ,  $35.58^\circ$ ,  $43.26^\circ$ ,  $53.83^\circ$ ,  $57.26^\circ$ ,  $62.80^\circ$ . The grain size or crystallite size ( $D$ ) of these NPs was calculated using Scherrer's formula [50], with a shape factor ( $\kappa$ ) of 0.95, X-ray beam wavelength ( $\lambda$ ) of  $1.548 \text{ \AA}$  for Cu radiation, FWHM (full width at half maximum) in radians of the most intense peak ( $\beta$ ) and Bragg angle ( $\theta$ ). The estimated sizes for different samples ranged between 13 and 21 nm.

$$D = \frac{\kappa\lambda}{\beta\cos\theta}$$



**Figure 1.** (a) XRD pattern of the synthesized MagNPs. (b) SEM image illustrates the uniform spherical shape of  $\text{Fe}_3\text{O}_4$ -NPs with an average size of about 23 nm. (c) DLS analysis for the synthesized NPs.

On the other hand, the SEM images of the synthesized MagNPs showed that the NPs were uniformly spherical in shape with an average size of about 23 nm, consistent with the values obtained from the powder XRD analysis, as depicted in Figure 1b.

Furthermore, DLS analysis showed that the average hydrodynamic diameter size of these NPs in water was  $156 \pm 14 \text{ nm}$ , indicating that water molecules surround the NPs in

solution, as shown in Figure 1c. Moreover, the zeta potential was measured and found to be negative at  $-31.6 \pm 13.7$  mV. This negative charge on the surface of these NPs shows that the aqueous solution has good potential to keep the particles suspended for a long time.

### 3.1. Antibacterial Activity of the Combination of MagNPs + Plants Extract

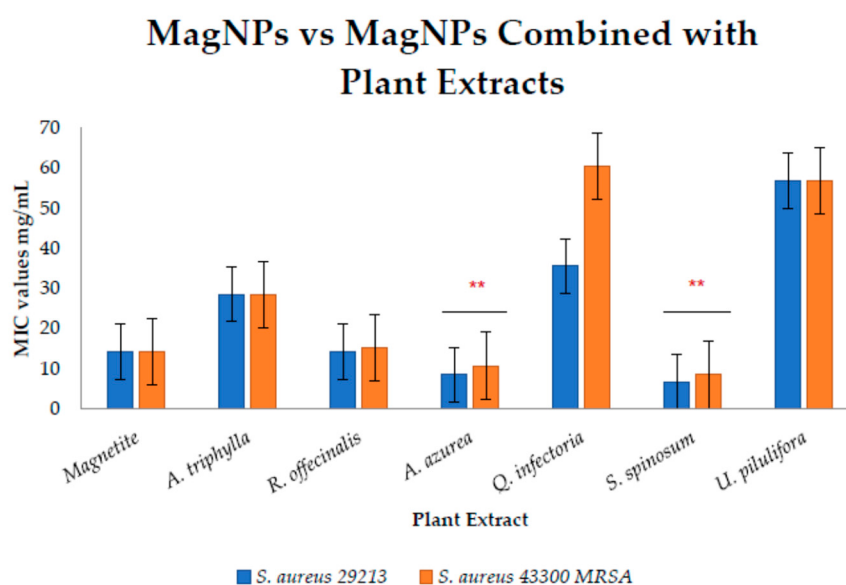
MIC and MBC tests were performed for each plant extract and MagNPs according to CLSI standards. Sub-MICs were then combined for 18–24 h for each plant with MagNPs before performing the test. The results are recorded for comparison and presented in Table 1 and Figures A1–A7 for all agents as MIC values in mg/mL for each examined strain. The MIC values for chloramphenicol antibiotics are consistent with those reported in the CLSI 30th edition (Performance Standards for Antimicrobial Susceptibility Testing, 2020), showing 4.0 µg/mL for both *S. aureus* strains and 2.0 µg/mL for both *E. coli* strains (Table 1).

The results indicate a significant increase in the effectiveness of three plant extracts when combined with MagNPs against the four strains used: *A. triphylla* (Figure A1), *S. spinosum* (Figure A5), and *U. pilulifera* (Figure A6), with *p*-values of: 0.0002, <0.0001, and 0.01, respectively. The effective concentration is reduced when the plant is combined with MagNPs.

In contrast, *R. officinalis*, *A. azurea*, and *Q. infectoria* extracts show notable effects against both *S. aureus* wild type and MRSA strains when combined with MagNPs but not against *E. coli* strains. While *Q. infectoria* extract alone did not impact MDR *E. coli* BAA 2452, when combined with MagNPs, 32 mg/mL of *Q. infectoria* extract inhibited the growth of the MDR strain *E. coli* BAA 2452 (Figure A4).

Figure 2 illustrates the evaluation of the MIC values of MagNPs alone and in combination with plant extracts to assess their impact. It was observed that MagNPs combined with *A. azurea*, and *S. spinosum* extracts had considerable effects against both *S. aureus* strains, with *p*-value of 0.001 and <0.001, respectively. Additionally, MagNPs combined with *A. azurea* extract alone had a substantial effect against *S. aureus* ATCC 29213. Conversely, *S. spinosum* extract combined with MagNPs was significantly effective against both wild type *S. aureus* and MRSA strains.

In conclusion, MRSA and MDR *E. coli* strains used in this study are highly affected by MagNPs, which combined with *A. triphylla*, *S. spinosum*, and *U. pilulifera* extracts significantly reduced the required concentrations compared to using the plant extracts alone. Furthermore, MagNPs showed a notable effect when combined with *S. spinosum* extract against MRSA strains compared to MagNPs alone.



**Figure 2.** Comparison between MICs of MagNPs values alone and combined with plant extracts against *S. aureus*, both strains. (\*\*) indicate *p*-value < 0.01.

MBC results were determined based on the number of colonies grown on plates compared to the positive control. The concentration resulting in fewer than five colonies on average was considered the MBC. The MIC and MBC values are presented in Table 1.

**Table 1.** MIC and MBC values of chloramphenicol  $\mu\text{g/mL}$ , MagNPs alone  $\text{mg/mL}$ , plant extract alone  $\text{mg/mL}$ , and plant extract combined with MagNPs  $\text{mg/mL}$  against *S. aureus* and *E. coli* wild type and MDR strains. MIC values represented by mean of 9 reads  $\pm$  standard deviation.

| #  | Agents                                  | Conc. $\text{mg/mL}$ | <i>S. aureus</i> ATCC 29213      |            | <i>S. aureus</i> ATCC 43300<br>MRSA |            | <i>E. coli</i> ATCC 25922         |             | <i>E. coli</i> BAA 2452<br>MDR    |             |
|----|-----------------------------------------|----------------------|----------------------------------|------------|-------------------------------------|------------|-----------------------------------|-------------|-----------------------------------|-------------|
|    |                                         |                      | MIC                              | MBC        | MIC                                 | MBC        | MIC                               | MBC         | MIC                               | MBC         |
| 1  | Chloramphenicol<br>( $\mu\text{g/mL}$ ) |                      | $4.0 \pm 0.0$                    | 4.0        | $4.0 \pm 0.0$                       | 4.0        | $2.0 \pm 0.0$                     | 2.0         | $2.0 \pm 0.0$                     | 4.0         |
| 2  | MagNPs                                  |                      | $14.22 \pm 3.33$                 | N          | $14.22 \pm 3.33$                    | N          | NE                                | -           | NE                                | -           |
| 3  | <i>A. triphylla</i>                     |                      | $0.5 \pm 0.0$                    | 0.5        | $1.06 \pm 0.4$                      | 1.0        | $32 \pm 0.0$                      | 64.0        | $32 \pm 0.0$                      | 64.0        |
| 4  | <i>A. triphylla</i> + MagNPs            |                      | <b><math>0.22 \pm 0.1</math></b> | 1.0        | <b><math>0.22 \pm 0.1</math></b>    | 1.0        | <b><math>16 \pm 0.0</math></b>    | 64          | <b><math>16 \pm 0.0</math></b>    | 64          |
| 5  | <i>R. officinalis</i>                   |                      | $0.56 \pm 0.2$                   | 1.0        | $0.47 \pm 0.1$                      | 0.5        | $5.33 \pm 1.9$                    | 16.0        | $4.89 \pm 1.7$                    | N           |
| 6  | <i>R. officinalis</i> + MagNPs          |                      | $0.06 \pm 0.0$                   | 0.25       | $0.06 \pm 0.0$                      | 0.25       | $8 \pm 0.0$                       | 32          | $32 \pm 0.0$                      | 64          |
| 7  | <i>A. azurea</i>                        |                      | <b><math>1 \pm 0.0</math></b>    | <b>2.0</b> | <b><math>0.53 \pm 0.2</math></b>    | <b>2.0</b> | <b><math>32 \pm 0.0</math></b>    | <b>64.0</b> | <b><math>32 \pm 0.0</math></b>    | <b>64.0</b> |
| 8  | <i>A. azurea</i> + MagNPs               |                      | $0.26 \pm 0.1$                   | 1.0        | $0.33 \pm 0.1$                      | 1.0        | $28.44 \pm 6.7$                   | N           | $32 \pm 0.0$                      | N           |
| 9  | <i>Q. infectoria</i>                    |                      | $8 \pm 0.0$                      | 8.0        | $7.11 \pm 1.7$                      | 8.0        | $32 \pm 0.0$                      | N           | NE                                | -           |
| 10 | <i>Q. infectoria</i> + MagNPs           |                      | $2.22 \pm 0.6$                   | 4.0        | $3.78 \pm 0.6$                      | 4.0        | $32 \pm 0.0$                      | 64          | $32 \pm 0.0$                      | 64          |
| 11 | <i>S. spinosum</i>                      |                      | $4.44 \pm 1.23$                  | 8.0        | $0.67 \pm 0.2$                      | 1.0        | $32 \pm 0.0$                      | N           | $32 \pm 0.0$                      | N           |
| 12 | <i>S. spinosum</i> + MagNPs             |                      | <b><math>0.42 \pm 0.1</math></b> | 1.0        | <b><math>0.13 \pm 0.1</math></b>    | 1.0        | <b><math>4.89 \pm 1.7</math></b>  | 16          | <b><math>8 \pm 0.0</math></b>     | 16          |
| 13 | <i>U. pilulifera</i>                    |                      | $16 \pm 0.0$                     | 32         | $8 \pm 0.0$                         | 32         | $32 \pm 0.0$                      | N           | $32 \pm 0.0$                      | N           |
| 14 | <i>U. pilulifera</i> + MagNPs           |                      | <b><math>7.11 \pm 1.7</math></b> | 16         | <b><math>7.11 \pm 1.7</math></b>    | 16         | <b><math>19.56 \pm 6.7</math></b> | N           | <b><math>30.22 \pm 5.0</math></b> | N           |

MIC: minimal inhibitory concentration  $\text{mg/mL}$ , MBC: minimal bactericidal concentration  $\text{mg/mL}$ , NE: not effective at the tested concentration, N: not detected/not bactericidal. Values in bold font are significant:  $p\text{-value} \leq 0.01$ .

### 3.2. Antibiofilm Assay

A biofilm formation assay was performed to confirm the ability of the tested strains to form biofilms, using the crystal violet quantification assay. The results were as follows: *E. coli* ATCC 25922 did not form biofilms, *E. coli* BAA 2452 formed weak biofilms, *S. aureus* ATCC 29213 formed moderate biofilms, and *S. aureus* ATCC 43300 formed strong biofilms, in addition to the positive control strain, *S. aureus* ATCC 33591. The obtained results are shown in Table A2. The calculated value for the OD cutoff value =  $0.095 + 3 \times 0.006 = 0.113$ .

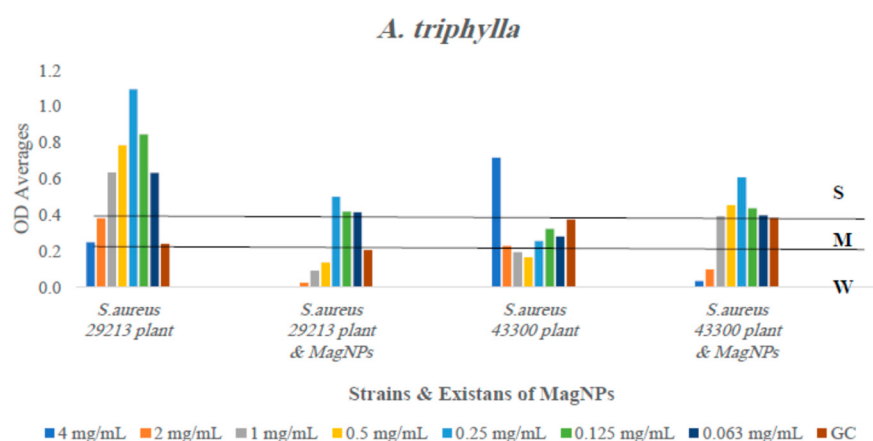
$$2 \times \text{OD cutoff value} = 0.226 \text{ and } 4 \times \text{OD cutoff value} = 0.452.$$

Both strains of *S. aureus* were subjected to the antibiofilm formation assay, while the weak biofilm former *E. coli* BAA 2452 was excluded from this test. Sub-MIC values were used for testing plants alone, MagNPs alone, and the combination assay. The results of plant extracts, MagNPs, and their combination with MagNPs agents against biofilm formation in *S. aureus* strains are illustrated in Figures 3–9 after subtracting the OD of the reference plate from the OD results.

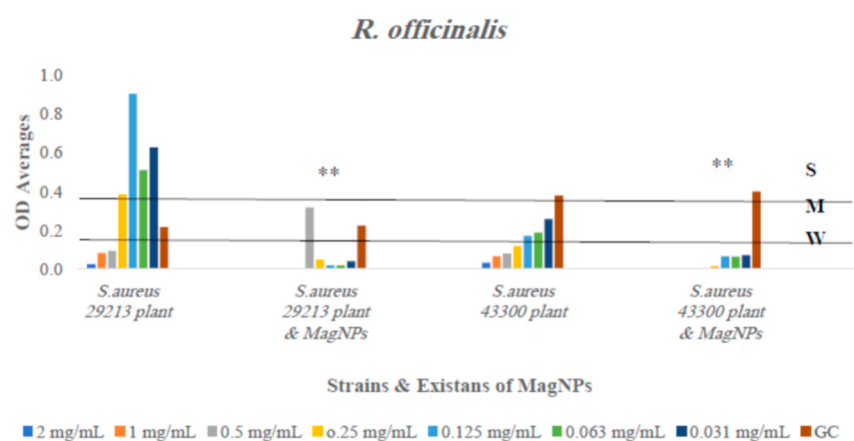
The outcomes showed inhibition in biofilm formation for four out of six plant extracts used when combined with MagNPs: *R. officinalis*, *A. azurea*, *Q. infectoria*, and *U. pilulifera* extracts significantly reduced the formation of biofilm by varying percentages against

both strains of *S. aureus* at all concentrations used with  $p$ -values of 0.0006, <0.0001, 0.003, and <0.0001, respectively. This indicates that the presence of MagNPs prevents biofilm development. However, the combination between *Q. infectoria* extract and MagNPs reduced biofilm development at lower concentrations more effectively than at higher concentrations.

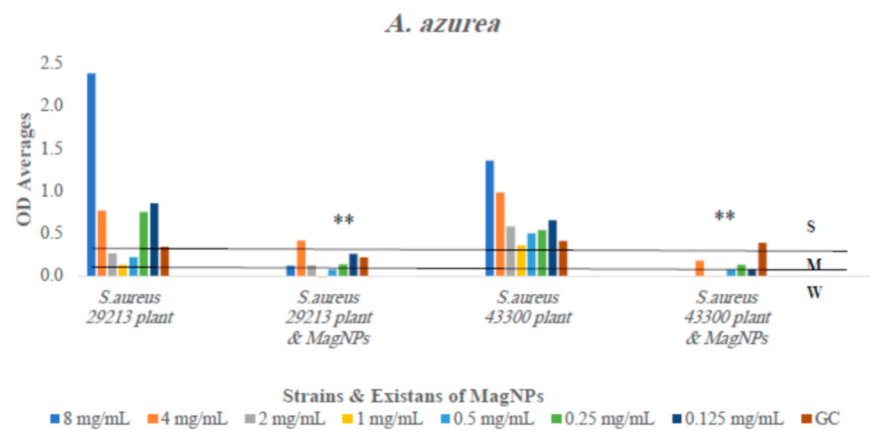
In contrast, *A. triphylla*, as illustrated in Figure 3, inhibited biofilm development in the wild-type strain *S. aureus* ATCC 29213 at all concentrations. We observed weakened biofilm formation at the higher three concentrations, and the two concentrations used against the wild type and MRSA, respectively, when *A. triphylla* extract was combined with MagNPs. Regarding *S. spinosum* extract, as shown in Figure 7, biofilm formation reduction occurred against the wild type strain in the presence of the plant extract alone, not in combination with MagNPs. Biofilm development for the MRSA strain was drastically inhibited at the lowest three concentrations of *S. spinosum* extract combined with MagNPs.



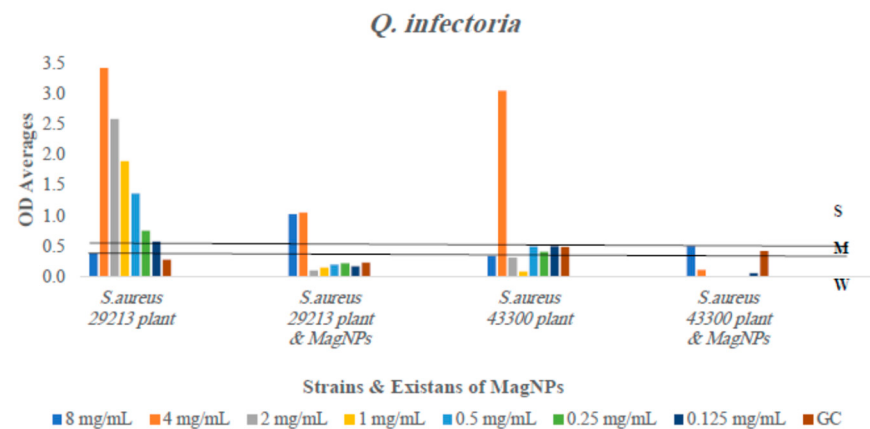
**Figure 3.** The antibiofilm formation values for *A. triphylla* extract alone and *A. triphylla* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, compared to the GC: strain alone.



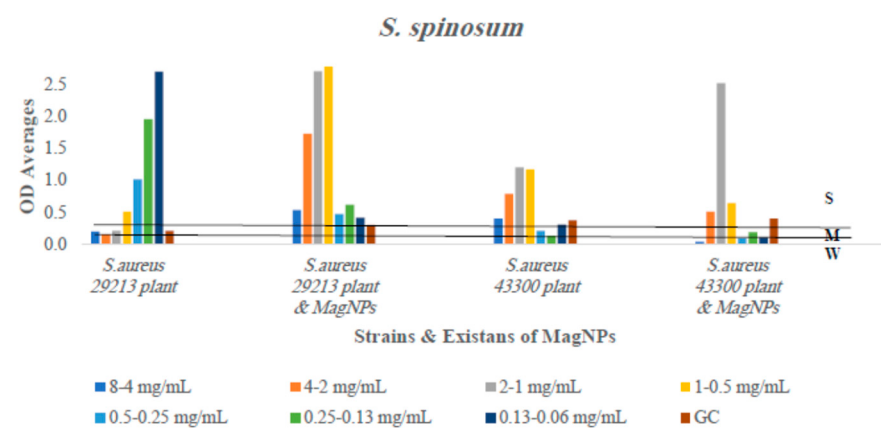
**Figure 4.** The antibiofilm formation values for *R. officinalis* extract alone and *R. officinalis* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, compared to the GC: strain alone. (\*\*) indicate  $p$ -value  $\leq 0.01$ .



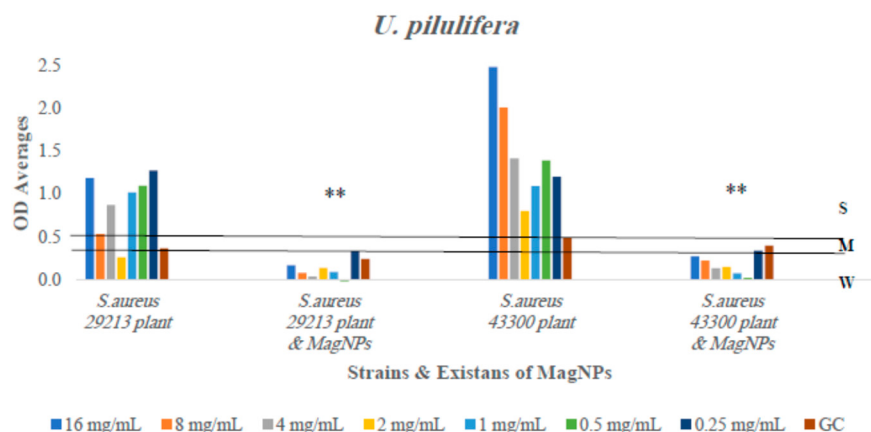
**Figure 5.** The antibiofilm formation values for *A. azurea* extract alone and *A. azurea* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, compared to the GC: strain alone. (\*\*) indicate  $p$ -value  $\leq 0.01$ .



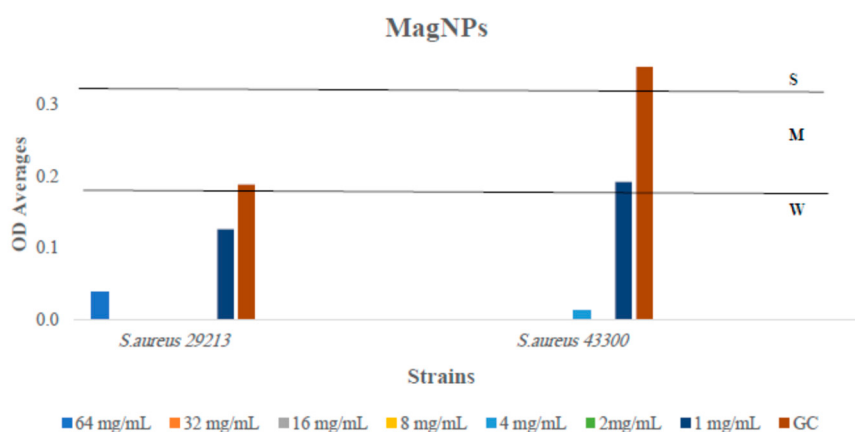
**Figure 6.** The antibiofilm formation values for *Q. infectoria* extract alone and *Q. infectoria* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, compared to the GC: strain alone.



**Figure 7.** The antibiofilm formation values for *S. spinosum* extract alone and *S. spinosum* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, the 1st number for 29213 strain and the 2nd for 43300 strain, compared to the positive control.



**Figure 8.** The antibiofilm formation values for *U. pilulifera* extract alone and *U. pilulifera* extract combined to MagNPs against both *S. aureus* strains presented as average OD readings from the crystal violet quantitative assay. The colors indicate the different concentrations used, compared to the GC: strain alone. (\*\*) indicate  $p$ -value  $\leq 0.01$ .



**Figure 9.** The antibiofilm formation values for MagNPs against both *S. aureus* strains presented as OD averages reads from crystal violet quantitative assay after subtracting the optical density (OD) readings obtained from MagNP e alone (reference plate). The colors indicate the different concentrations used, compared to the GC: strain alone.

In conclusion, MagNPs significantly enhance the inhibition of biofilm development at various concentrations used in this study for both assessed strains when tested in combination with *R. officinalis*, *A. azurea*, *Q. infectoria*, and *U. pilulifera* compared to the use of the plant extract alone.

#### 4. Discussion

Bacterial resistance has become a global challenge that requires innovative solutions to combat this problem. In the present study, we investigated the antibacterial effect of MagNPs combined with plant extracts with known antibacterial properties. MagNPs were chosen for their proven inhibitory effect, whether alone or when stabilized with various materials against different pathogens, as well as their low toxicity and biocompatibility with living cells. Plant methanolic extracts were chosen as a valuable source of secondary metabolites, offering a cost-effective and potentially low-side-effect alternative against resistant bacteria. Therefore, we evaluated this novel combination against *S. aureus* and *E. coli* wild type and MDR ATCC strains.

The coprecipitation method, used with continuous sonication, proves to be an efficient approach to producing MagNPs that are uniformly spherical, as demonstrated in SEM images, with an average size of 16 nm as determined by XRD calculations. The smaller

size of the MagNPs enhances their efficacy as antibacterial agents. The XRD patterns of the MagNPs in this study are consistent with those in previous studies, validating the reliability of the coprecipitation method with continuous sonication in producing well defined MagNPs. Our findings align with studies by Petcharoen et al. (2012), Alomari et al. (2015), and Saqib et al. (2018) [51–53], despite variations in procedural details.

The use of continuous sonication during the synthesis of MagNPs, as employed in the current study, was notably mentioned only in the work of Alomari et al., whereas previous studies on MagNPs primarily focused on stirring without sonication during the coprecipitation procedure [41,42,51,54]. According to Ahn et al. (2012), the mixing step during MagNPs synthesis is critical for producing uniformly spherical MagNPs with a small size and well defined structure [55]. These characteristics render MagNPs a promising option as an antibacterial agent.

The present findings show that MagNPs alone affected *S. aureus* strains ATCC 29213 and MRSA ATCC 43300 at an MIC of about 15 mg/mL, consistent with the results of Tran et al. Additionally, PVA-coated MagNPs ( $9 \pm 4$  nm in size) affected the sensitive strain of *S. aureus* at 3 mg/mL all tested duration times [24]. However, despite previous studies indicating that MagNPs affect different strains of *E. coli*, including those by Auffan et al. and Al-Shabib et al., the synthesized MagNPs in this study did not affect the *E. coli* wild type or MDR strains used [21,56]. Further studies are needed to explore the mechanism of action of the MagNPs against different bacterial strains and to improve their usage as antimicrobial agents.

The current study demonstrates that all the plant methanolic extracts inhibit the growth of the tested strains. The inhibition varied depending on the plant extract, the concentration used, and the bacterial strain. A broad spectrum of antibacterial activity was exhibited by *A. triphylla*, *R. officinalis*, *A. azurea*, *S. spinosum*, and *U. pilulifera*, specifically against MRSA and MDR *E. coli*. *Q. infectoria* bark extract inhibited wild type *E. coli* but not the MDR strain.

*A. triphylla* methanolic extracts inhibit the growth of *S. aureus*-sensitive MRSA, *E. coli* wild type, and MDR strains with MIC values of 0.5, 1.0, 32.0, and 32.0 mg/mL, respectively. These results are in agreement with the findings of Hanaa et al. (2011) using the essential oil of *A. triphylla* and Batatineh et al. (2019) using methanolic extract [57,58]. On the other hand, *R. officinalis* essential oil exhibited good antibacterial activity against wild type strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, with MIC values of 4 and 3.5 mg/mL, respectively. The current study shows that crude methanolic extracts of *R. officinalis* were effective at 0.5 and 5.0 mg/mL on *S. aureus* and *E. coli* strains, respectively, as well as on MRSA and MDR strains at 0.5 and 5.0 mg/mL.

The current study also investigated the antibacterial activity of *Q. infectoria* bark methanolic extract. It is worth mentioning that *Q. infectoria* nut gall extract has been extensively studied for its antibacterial effect [59]. However, *Q. infectoria* bark extract has received less research attention for its antibacterial activity, especially against MDR bacteria. The present findings showed that a methanolic extract of *Q. infectoria* bark inhibited *S. aureus* against both strains by 8 mg/mL; however, the wild type *E. coli* MIC was inhibited by 32 mg/mL with no effect on the MDR *E. coli* strain. *S. spinosum* root methanolic extract exhibits substantial activity against all the tested strains compared to aqueous and ethanolic *S. spinosum* extracts, which have been shown to have activity against wild type strains of *S. aureus* and *E. coli* [45].

The combination of plant extract and MagNPs boosted the effectiveness of both plant extract and MagNPs against the examined strains, including MDR strains. Exceptional inhibition was observed against the MRSA strain when using the combination of MagNPs with all plants. In addition to the *S. aureus* strains examined, *E. coli* wild type and MDR strains were significantly inhibited when treated with *A. triphylla*, *S. spinosum*, and *U. pilulifera* extracts. In contrast, MagNPs synthesized using *R. officinalis* aqueous extract produced 1–12 nm particles and exhibited antibacterial activity against *E. coli* PTCC 1330 at a concentration of 250 µg/mL [60]. A recent study successfully synthesized iron oxide NPs from *Zingiber officinale* (Ginger) with a size of 5.1 nm. The synthesized Iron oxide

NPs exhibited antibacterial activity against *E. coli* [61]. Arokiaraj et al. (2013) achieved comparable results using MagNPs combined with *Argemone mexicana* leaf ethanolic extract for 12 h against *E. coli*, *P. mirabilis*, and *B. subtilis* [28]. The synergistic effect of plant extract and MagNPs was achieved by combining sub-MICs for 24 h. However, no significant effect was observed at the MIC values of the plant extract. Therefore, we doubled the MIC and used  $2 \times \text{MIC}$  or  $4 \times \text{MICs}$ . *E. coli* typically employs multiple metabolic pathways to resist external agents, necessitating the use of higher concentrations of the plant extract when combined with MagNPs. The combination of MagNPs and plant extracts was efficient in preventing the development of biofilm in the resistant strain MRSA and the sensitive strain *S. aureus*. Among the plant extracts used, *R. officinalis*, *A. azurea*, *Q. infectoria*, and *U. pilulifera* methanolic extracts successfully reduced biofilm development. These findings are important in the challenge of bacterial resistance emergence, even though the combination had no effect against planktonic cells. Preventing the development of biofilm in resistant strains could provide scientists with an opportunity to overcome these infections.

The outcomes of this novel approach, combining MagNPs with plant extracts, align with the synergistic use of plant extracts with various antibiotics, NPs, or other therapeutic combinations. Therefore, these results suggest that alternatives to antibiotics can be explored to combat the emergence of antibiotic resistance. The combination of different materials is a promising new strategy for resistant bacterial infections. Thus, MagNPs have the advantage of magnetism, enabling their reproducibility, targeted delivery, and easy separation from fluids using an external magnet.

## 5. Conclusions

The urgent need to address antibiotic resistance drives research into plant extracts as a source of secondary metabolites and a safe alternative to fighting resistant bacteria. This work describes the effectiveness of combining different plant extracts with chemically synthesized MFeO-NPs against different strains of bacteria. Such investigations hold promise for their potential use in treating bacterial infections as an alternative to antibiotics. The plant extracts used in this study have several degrees of effectiveness against Gram-positive, Gram-negative strains, and wild type and resistant strains. When combined with MFeO-NPs, plant extracts of *A. triphylla*, *S. spinosum*, and *U. pilulifera* boosted the antibacterial growth against all examined strains: *S. aureus* and *E. coli*. Combining MagNPs with *R. officinalis*, *A. azurea*, *Q. infectoria*, and *U. pilulifera* extracts exhibits antibiofilm activity against both *S. aureus* strains. The combination of MagNPs with plant extracts inhibited biofilm development at least at two of the seven evaluated concentrations.

## 6. Recommendation and Future Work

The present study paves the way for additional experiments to determine the precise mechanisms of action for the effective combinations. One of the recommended future research directions is to fractionate the plant extract using phytochemical investigation combined with MagNPs. In addition, further testing using animal models and performing toxicity and safety analyses are also required. This could lead to novel drug synthesis and contribute to the development of effective treatments for antibiotic-resistant infections. Furthermore, *in vitro* analysis can be used to determine the specific mode of action of MagNPs against viable cells or biofilm-forming strains. Tracking specific molecules is recommended for understanding the mechanisms of action.

**Author Contributions:** Conceptualization, S.M.B.B. and I.M.A.; Methodology, S.M.B.B., I.M.A. and S.M.A.-Z.; Software, H.M.H.; Validation, S.M.B.B. and I.M.A.; Formal analysis, S.M.A.-Z.; Investigation, S.M.B.B., I.M.A. and S.M.A.-Z.; Resources, S.M.B.B., M.M.A.-G. and Y.H.T.; data curation, S.M.A.-Z. and H.M.H.; Writing—original draft, S.M.A.-Z.; Writing—review & editing, S.M.B.B., S.M.A.-Z., I.M.A., M.M.A.-G., H.M.H. and H.D.; Visualization, S.M.A.-Z., S.M.B.B. and I.M.A.; Supervision, S.M.B.B. and I.M.A.; Project administration, S.M.B.B.; Funding acquisition, S.M.B.B. All authors have read and agreed to the published version of the manuscript.

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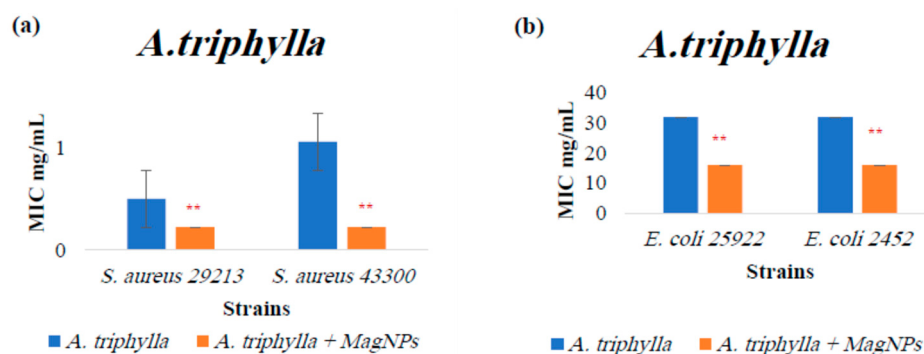
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**Conflicts of Interest:** The authors declare no conflicts of interest.

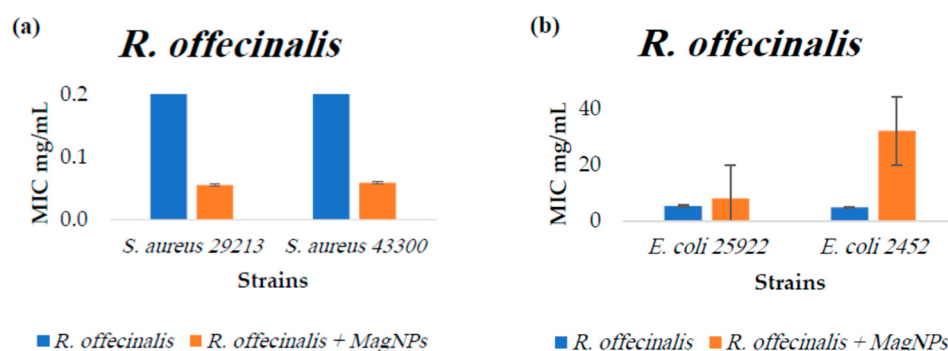
## Appendix A

**Table A1.** Plant's used parts with their local names.

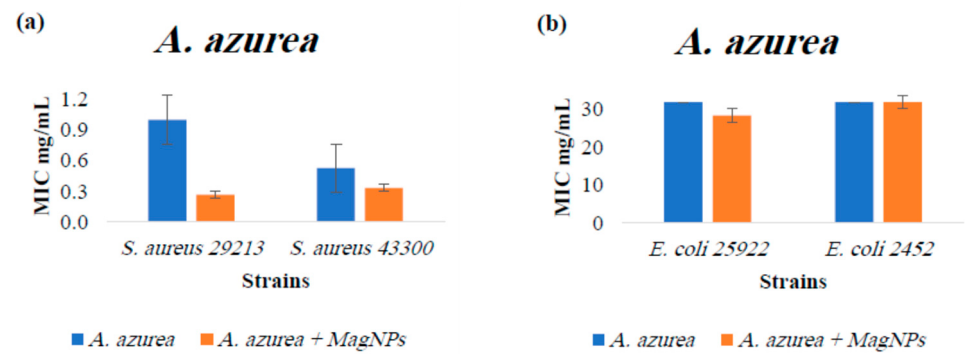
| Plant Name                    | Plant Type | Part Used    | Arabic Common Name    |
|-------------------------------|------------|--------------|-----------------------|
| <i>Rosmarinus officinalis</i> | Bush       | Aerial parts | Eklel El-Jabal        |
| <i>Aloysia triphylla</i>      | Bush       | Aerial parts | Mallisa               |
| <i>Anchusa azurea</i>         | Herb       | Aerial parts | Hamham/Lesan El-Thour |
| <i>Quercus infectoria</i>     | Tree       | Bark         | Dbbagh                |
| <i>Sarcopoterium spinosum</i> | Bush       | Roots        | Billan                |
| <i>Urtica pilulifera</i>      | Herb       | Aerial parts | Qarass                |



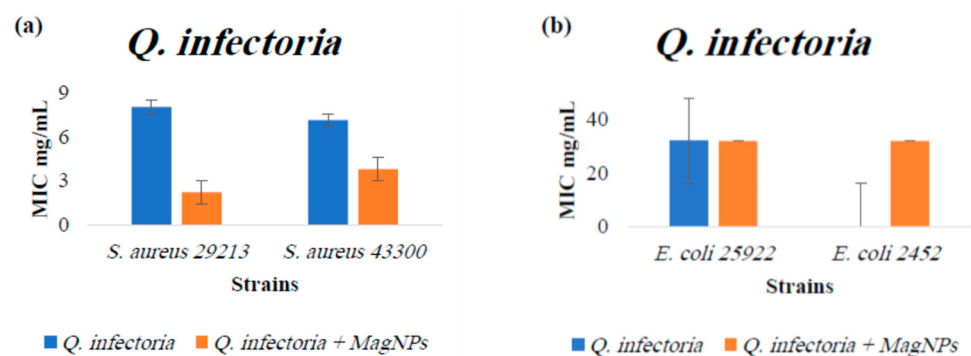
**Figure A1.** MIC values for *A. triphylla* extract alone and *A. triphylla* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. (\*\*) indicates  $p$ -value  $\leq 0.01$ . MIC values for chloramphenicol antibiotic as a positive control were: 4.0  $\mu\text{g/mL}$  for both *S. aureus* strains, and 2.0  $\mu\text{g/mL}$  for both *E. coli* strains.



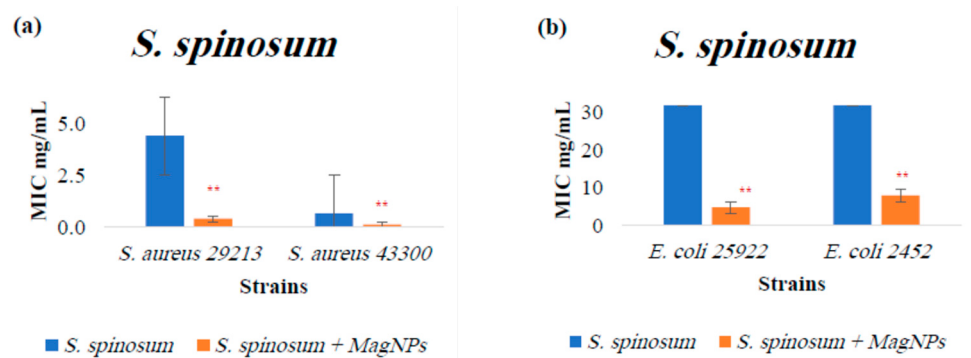
**Figure A2.** MIC values for *R. officinalis* extract alone and *R. officinalis* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. MIC values for chloramphenicol antibiotic as a positive control were: 4.0  $\mu\text{g/mL}$  for both *S. aureus* strains, and 2.0  $\mu\text{g/mL}$  for both *E. coli* strains.



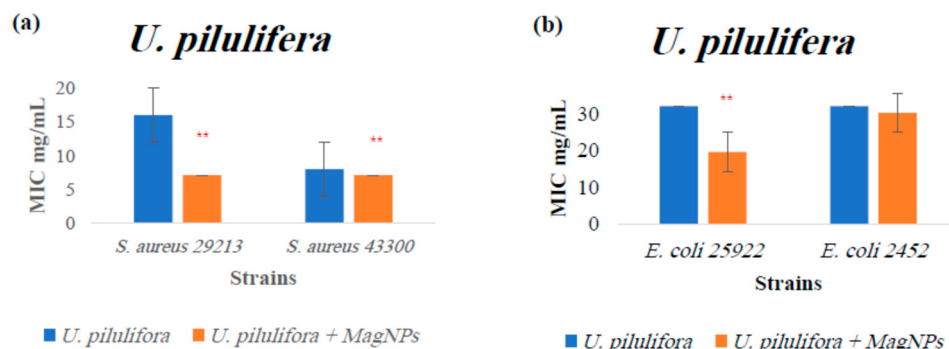
**Figure A3.** MIC values for *A. azurea* extract alone and *A. azurea* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. MIC values for chloramphenicol antibiotic as a positive control were: 4.0 µg/mL for both *S. aureus* strains, and 2.0 µg/mL for both *E. coli* strains.



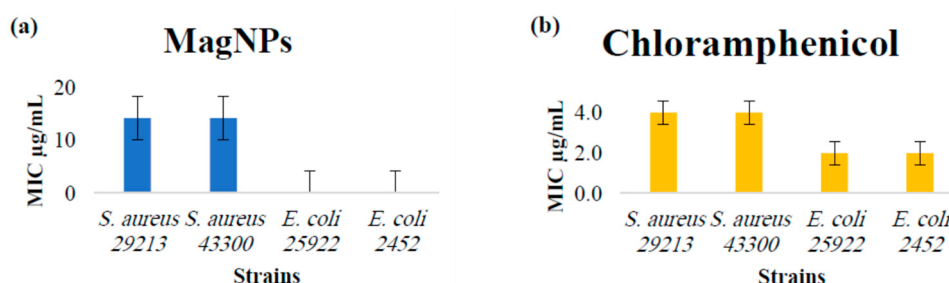
**Figure A4.** MIC values for *Q. infectoria* extract alone and *Q. infectoria* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. MIC values for chloramphenicol antibiotic as a positive control were: 4.0 µg/mL for both *S. aureus* strains, and 2.0 µg/mL for both *E. coli* strains.



**Figure A5.** MIC values for *S. spinosum* extract alone and *S. spinosum* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. (\*\*) indicates  $p$ -value  $\leq 0.01$ . MIC values for chloramphenicol antibiotic as a positive control were: 4.0 µg/mL for both *S. aureus* strains, and 2.0 µg/mL for both *E. coli* strains.



**Figure A6.** MIC values for *U. pilulifera* extract alone and *U. pilulifera* extract combined with MagNPs against (a): *S. aureus*, (b): *E. coli*. (\*\*) indicates  $p$ -value  $\leq 0.01$ . MIC values for chloramphenicol antibiotic as a positive control were: 4.0  $\mu\text{g/mL}$  for both *S. aureus* strains, and 2.0  $\mu\text{g/mL}$  for both *E. coli* strains.



**Figure A7.** (a) MIC values for MagNPs against *S. aureus*, *E. coli*. (b) MIC values for chloramphenicol antibiotic as a positive control were: 4.0  $\mu\text{g/mL}$  for both *S. aureus* strains, and 2.0  $\mu\text{g/mL}$  for both *E. coli* strains.

**Table A2.** The biofilm formation strength for the studied strains was assessed relative to the strength of biofilm formation determined by the negative control OD cutoff value, and contrasted with the positive control (a strong biofilm former strain). The classification used was as follows: N: non biofilm former, W: weak biofilm former, M: moderate biofilm former, and S: strong biofilm former. Negative control represented by mean  $\pm$  SD used to calculate OD cutoff value of the negative control.

| Bacterial Strain    | <i>S. aureus</i> ATCC 29213 | <i>S. aureus</i> ATCC 43300 MRSA | <i>E. coli</i> ATCC 25922 | <i>E. coli</i> BAA 2452 MDR | <i>S. aureus</i> ATCC 33591 Positive Control | Negative Control (TSB) |
|---------------------|-----------------------------|----------------------------------|---------------------------|-----------------------------|----------------------------------------------|------------------------|
| OD average          | 0.365                       | 0.527                            | 0.108                     | 0.193                       | 0.482                                        | 0.095 $\pm$ 0.006      |
| Strength of biofilm | M                           | S                                | N                         | W                           | S                                            | N                      |

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