ORIGINAL PAPER

Antimicrobial effects of *Glycyrrhiza glabra* extract, iron oxide nanoparticles, and *Lactobacillus rhamnosus* on a biofilm composed of *Pseudomonas aeruginosa* in glass, wood, and polysteel

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ABSTRACT

Biofilm formation is a pathogenicity factor of *Pseudomonas aeruginosa*, which causes inherent resistance to a wide range of antibiotics in the strains. The present study aimed to compare the inhibitory effects of *Glycyrrhiza glabra* **(**licorice) extract, iron oxide nanoparticles, and *Lactobacillus rhamnosus* suspension on a biofilm composed of *P. aeruginosa* in various levels of glass, wood, and polysteel. This descriptive, cross-sectional study assessed the effects of *Glycyrrhiza glabra* extract, iron oxide nanoparticles, and *Lactobacillus rhamnosus* suspension on the standard biofilm of *P. aeruginosa* 1601PTCC on glass, steel, and wood surfaces. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also calculated. The obtained results showed that each antimicrobial agent had different effects on *P. aeruginosa*, and the MIC and MBC exerted inhibitory properties. In addition, the largest inhibition zone diameter was 28 mL due to the effect of the *Glycyrrhiza glabra* extract on free bacteria in the volume of 180 microliters, and the highest inhibitory level was observed on the polysteel and glass surfaces with the inhibition zone diameter of 20-20.66 millimeters in the volume of 180 microliters. The highest inhibition in the bacterial biofilm was observed on the polysteel surface, and a significant difference was also denoted in this regard with the glass and wood surfaces ($P<0.05$). Therefore, it could be concluded that licorice (*Glycyrrhiza glabra* L.) had more significant antimicrobial properties compared to the iron oxide nanoparticles and *Lactobacillus rhamnosus* suspension.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Iron oxide nanoparticles, *Glycyrrhiza glabra* extract, *Lactobacillus rhamnosus*

Introduction

Pseudomonas aeruginosa is an important causative agent of infection in humans, especially nosocomial infections. It is an aerobic, gram-negative, opportunistic pathogen and a major cause of death in immunocompromised patients due to life-threatening infections.[1, 2] *P. aeruginosa* is highly aerobic and uses oxygen molecules as the final electron receptor.[3] Biofilm formation is a pathogenicity factor of this bacterium.[4] Biofilms are structures composed of a set of bacteria, which are in a polymer matrix produced by the bacteria themselves and are able to bind to different surfaces.[5] This natural law has made these microorganisms resistant to antibiotics and led to the emergence of resistant strains.[4] To solve the basic issue against antibiotic-resistant microorganisms, the antimicrobial properties of nanoparticles, herbal extracts, and probiotics have been extensively investigated.[6]

Metal oxide nanoparticles (e.g., iron oxide) could be an effective alternative to

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antibiotics in the treatment of bacterial infections considering their antimicrobial potential.[7-9] Iron oxide nanoparticles in small concentrations can act as a source of iron ions required by microorganisms, while their high concentrations may bind to the bacterial membrane through electrostatic reaction and cause interference and destruction.[8] Researchers have concluded that metal oxide nanoparticles are highly active and have remarkable bactericidal activity against gram-positive and gram-negative bacteria.[10]

Glycyrrhiza glabra rhizome are reported to have therapeutic and medicinal properties owing to the high concentration of saponins and other compounds, and the antimicrobial activity of saponins against numerous disease-causing agents has been confirmed.[11] Probiotics are living, non-pathogenic human microorganisms with beneficial health effects on the host through affecting the microbial flora of humans and animals.[12] Probiotics are mainly of the genus *Bifidobacterium lactobacillus*.[13] Probiotic bacteria with the ability of binding and cell aggregation inhibit the binding of pathogenic bacteria, and the production of compounds such as proteins could identify pathogenic bacteria. *Pseudomonas aeruginosa* is found in various levels of wood, glass, and polystyrene.

The present study aimed to compare the antimicrobial properties of iron oxide nanoparticles and *Glycyrrhiza glabra* extract and *Lactobacillus rhamnosus* suspension on a biofilm composed of *P. aeruginosa* on various surfaces of wood, glass, and polysteel.

Materials and Methods

Preparation of bacterial strains

This descriptive, cross-sectional study was conducted in 2017. The experimental bacteria included the standard strains of *P. aeruginosa* ATCC7853 and *L. rhamnosus* PTCC1601, which were obtained from a Persian culture collection in the lyophilized form and purchased from the Institute of Biotechnology Research of the University of Tehran, Iran. The bacterial strains were thawed at room temperature, and after culturing in tryptic soy broth (TSB), incubated at the temperature of 37 °C for 24 h.

Preparation of nanoparticles

Iron oxide nanoparticles with the purity of 99% and particle size of 20 nanometers were purchased from Pishgaman Materials Iranian Company (Mashhad, Iran). To disperse the nanoparticles in the culture medium, the nanoparticle solution was placed in an ultrasonic bath for 20 min on a shaker before use. The rhizome of *Glycyrrhiza glabra* was collected from the northwest of Iran, identified, and confirmed by the Department of Agricultural Research. After drying at the appropriate temperature and shade, 100 grams of the plant was ground, and extraction was performed using the percolation method with 85% methanol solvent. After extraction, the solvent was evaporated using an oven and prepared for diffusion inside the wells.[14]

Preparation of the probiotic supernatant

In order to prepare the cell culture supernatants from *L. rhamnosus*, a fresh culture was initially prepared in MRS agar and broth media, and the culture medium was centrifuged at the temperature of 25 °C and 10,000 rpm and filtered (0.4 μm) to ensure the absence of microbial cells.[15]

Formation of P. aeruginosa biofilm on wood, glass, and polysteel surfaces

At the first stage, the 18-24-hour culture of *P. aeruginosa* was prepared in a plate containing 2% TSA glucose medium (Merck, Germany) and incubated at the temperature of 37 °C. Following that, the single colonies grown on the medium were collected to form a biofilm and brought to the turbidity of 0.5 McFarland. One milliliter of the formed biofilm was added to an Erlenmeyer flask containing 50 milliliters of sterile TSB and 0.2% glucose, as well as several slides of disinfected glass, wood, and polyethylene surfaces. The Erlenmeyer was shaken at 100 rpm in the laboratory at room temperature and incubated. After 18-28 hours, the slides were taken out of the Erlenmeyer. To sterilize the

polysteel, glass, and wood surfaces, coupons (3×3 cm) were prepared, and the steel and glass coupons were immersed in 96% methanol and rinsed three times with double distilled water. Finally, the coupons were autoclaved with distilled water. Notably, the wood coupons were not soaked in the solvent due to the permeability of the wood tissues and only disinfected in an autoclave.[16]

Antimicrobial effects of iron oxide nanoparticles and Glycyrrhiza glabra extract

In order to investigate the susceptibility of the bacteria to the iron oxide nanoparticles, the *Glycyrrhiza glabra* extract, and *L. rhamnosus* suspension, *P. aeruginosa* microbial suspension of 0.5 McFarland, which had resulted from the bacterial biofilm, was prepared on different surfaces using a sterile culture medium. The Müller-Hinton Agar (Merck, Germany) was also used as the glass culture. A sterile pipette was used to prepare wells with the diameter of 10 nanometers on the mentioned medium. The wells were inoculated with the iron oxide nanoparticles (100, 150, and 180 µL) at the concentration of 0.02 g/mL, *Glycyrrhiza glabra* extract at the concentration of 0.2 g/mL, and *L. rhamnosus* suspension. Each plate had positive and negative controls; the positive control wells contained the microbial suspension and culture medium, and the negative control wells contained the bacterial culture medium only. The plates were incubated at the temperature of 37 °C for 24 h. Afterwards, the inhibition zone diameter was measured using a caliper, and the experiment was performed in triplicate.

The tube dilution method was applied ensure the minimum bacterial growth inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). To this end, 1,000 microliters of the licorice extract samples and *L. rhamnosus* suspension was loaded into tube No. 1, and after mixing, dilution was performed up to tube No. 9. Finally, 1,000 microliters was collected from tube No. 9, and 100 microliters of 0.5 McFarland of the active bacteria was added to each dilution per milliliter of the liquid medium in the tube. The samples were incubated for 24 h at the temperature of 37 °C. To determine the MBC, 0.5 mL was collected from all the tubes without turbidity and cultured on the Müller-Hinton agar medium. The last dilution of the *Glycyrrhiza glabra* extract, iron oxide nanoparticles, and *L. rhamnosus* suspension was able to kill 99.9% of the primary living bacteria and considered as the culture. The same steps were performed to determine the MIC and MBC biofilm of *P. aeruginosa* on the wood, glass, and polysteel surfaces separately.

Data analysis was performed in SPSS version 25 using one-way analysis of variance (ANOVA), and the P-value of less than 0.05 was considered significant.

Results and Discussion

In the present study, we investigated the antimicrobial effects of the iron oxide nanoparticles, *Glycyrrhiza glabra* extract, and *L. rhamnosus* suspension on the biofilm composed of *P. aeruginosa* on the surfaces of wood, glass, and polystyrene.

Effects of Glycyrrhiza glabra extract on P. aeruginosa and its biofilm

The results of the inoculation of the *Glycyrrhiza glabra* extract on the bacterium and its biofilm indicated that the antimicrobial substance had inhibitory properties. In addition, the inhibition zone diameter was observed in the bacterium and its biofilm. The largest inhibition zone diameter through the effect of the *Glycyrrhiza glabra* extract on the free bacteria was 28 millimeters in the volume of 180 microliters, and the highest inhibitory level was observed on the polysteel and glass surfaces where the inhibition zone diameter was 20-20.66 millimeters in the volume of 180 microliters. Figs. 1 and 2 show the mean changes in the inhibition zone diameter of the prepared concentrations. Fig. 3 depicts the formed biofilm of *P. aeruginosa* on the wood, glass, and polystyrene surfaces. Accordingly, the rate of biofilm formation initially increased on the wood surface, followed by the glass and

polysteel surfaces. The statistical results indicated a significant difference between the three levels in this regard (P<0.05).

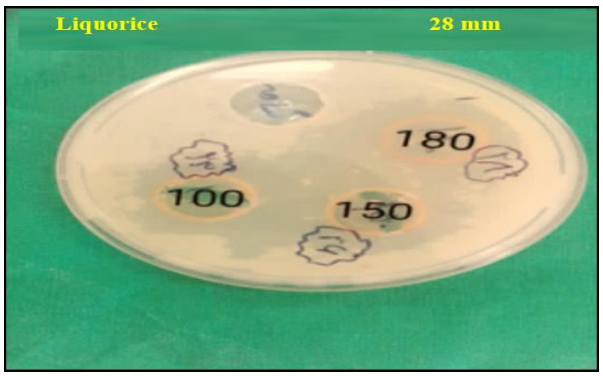


Fig. 1. Results of inoculation of *Glycyrrhiza glabra* extract in *Pseudomonas aeruginosa*

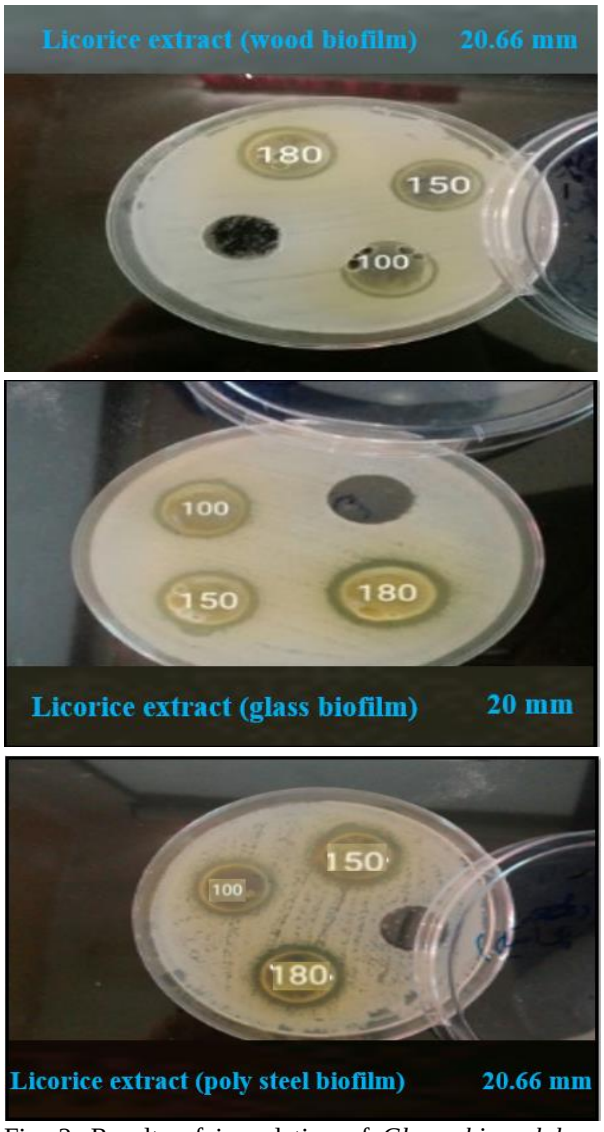


Fig. 2. Results of inoculation of *Glycyrrhiza glabra* extract in *Pseudomonas aeruginosa* bacterial biofilm on wood, glass, and polysteel surfaces

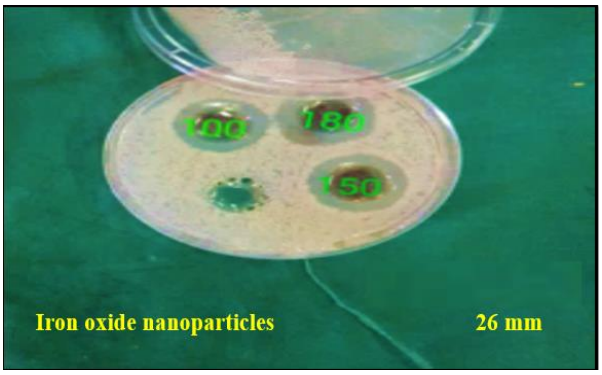


Fig. 3. Effects of iron oxide nanoparticles on *P. aeruginosa*

According to the results of the preset study, the well plate diffusion method along with the increased volume of the *Glycyrrhiza glabra* extract caused no increase in the inhibition zone diameter of the iron oxide nanoparticles and *L. rhamnosus* suspension. The statistical analysis indicated a significant difference in the antimicrobial substances of the *Glycyrrhiza glabra* extract, iron oxide nanoparticles, and *L. rhamnosus* suspension (P<0.05). Furthermore, the highest antimicrobial activity and inhibition zone diameter of the *Pseudomonas* bacteria belonged to the free bacteria, and a significant difference was also observed with the bacterial biofilm in this regard (P<0.05). This finding is consistent with the results obtained by Chakotiya *et al*.[17]

Effects of iron oxide nanoparticles on P. aeruginosa and its biofilm

After using the iron oxide nanoparticles at combined concentrations against *P. aeruginosa*, the obtained results indicated the production of the inhibition zone diameter and reduced number of the free bacterial cells and biofilms formed on the wood, glass, and polysteel surfaces. As is depicted in Fig. 3, the mean changes in the inhibition zone diameter in the planktonic state and the volume of 180 microliters was 26 millimeters, while Fig. 4 shows that it was 16-20.66 millimeters in the bacterial biofilm on the surfaces of wood, glass, and polysteel in the volume of 180 microliters. The other volumes were less than 180 microliters (Fig. 4), and a significant difference was observed between the three

levels (P<0.05). On the other hand, the highest level of inhibition was denoted on the polysteel surface, with a significant difference compared to the planktonic state of *P. aeruginosa*. Fig. 1 shows the changes in the inhibition zone diameter of different volumes in the planktonic and biofilm states (P<0.05). In a study in this regard, Salahvarzan *et al*. reported that *Glycyrrhiza glabra* had the highest antimicrobial activity against bacteria and yeasts, especially against *P. aeruginosa*.[11]

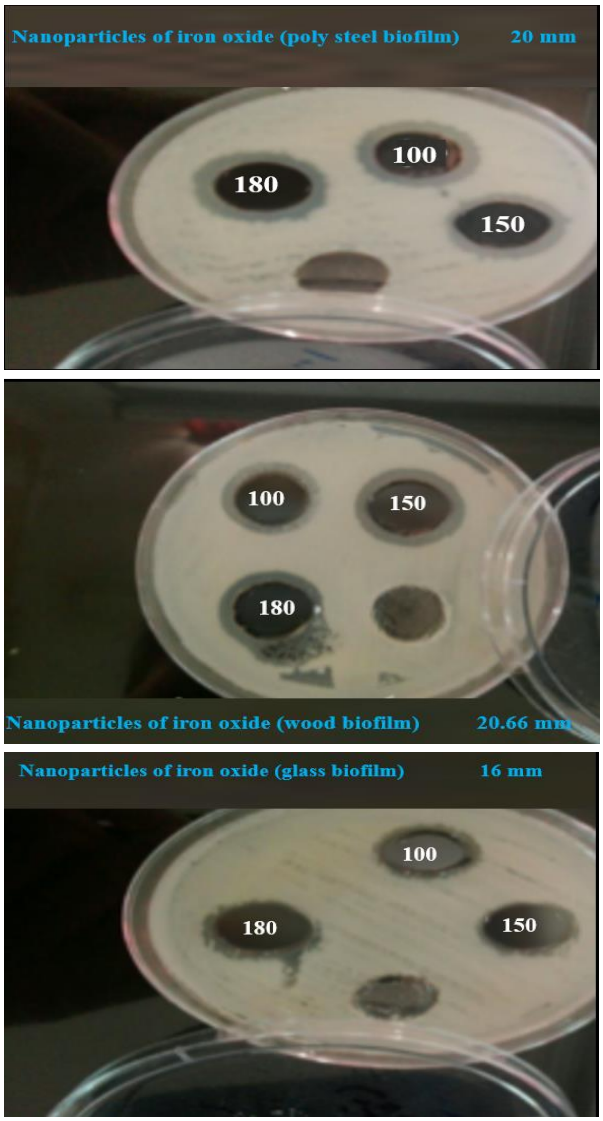


Fig. 4. Results of inoculation of iron oxide nanoparticles in *P. aeruginosa* biofilm formed on wood, glass, and polysteel surfaces (Numbers shown on arrow of figures show inhibition zone diameter in well with volume of 180 microliters)

Effects of L. rhamnosus suspension on P. aeruginosa and its biofilm

After using the suspension of the *L. rhamnosus* strain on *P. aeruginosa* and its biofilm, the well plate diffusion method was employed for evaluation. As is shown in Figs. 5 and 6, the suspension of *L. rhamnosus* was able to inhibit *P. aeruginosa* and the production of the inhibition zone by this bacterial strain in the free bacterial state, and the rate of biofilm formation on the surfaces of wood, glass, and polysteel at the volume of 180 microliters was estimated at 17.66-20.66. In addition, the growth inhibition rate of free *P. aeruginosa* increased compared to its biofilm, and the difference in this regard was considered significant (P<0.05).

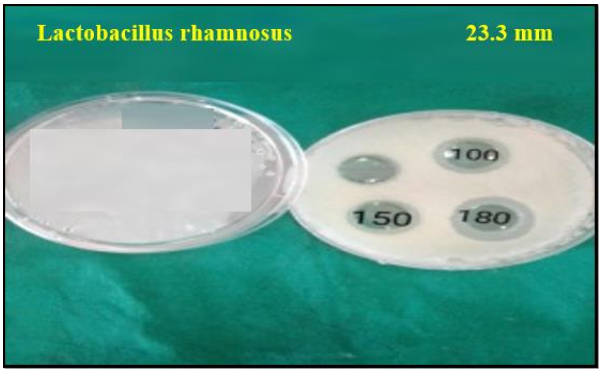
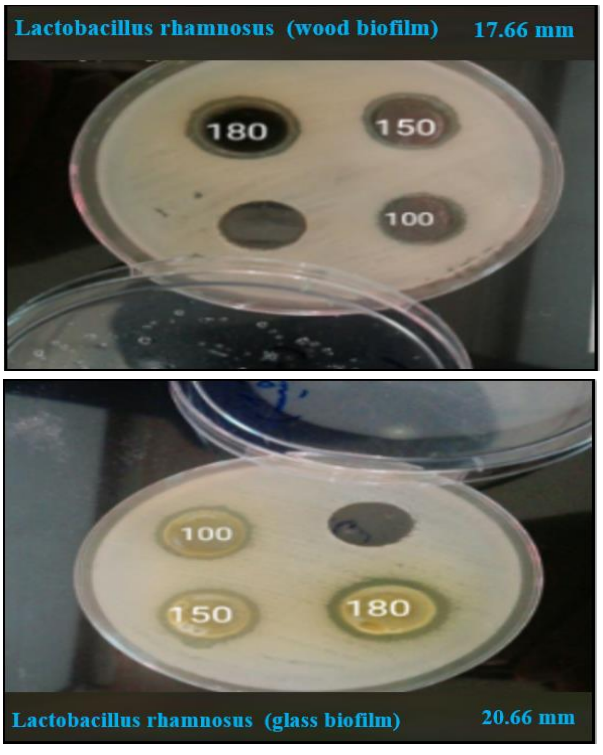


Fig. 5. Effect of *L. rhamnosus* probiotic on *P. aeruginosa*



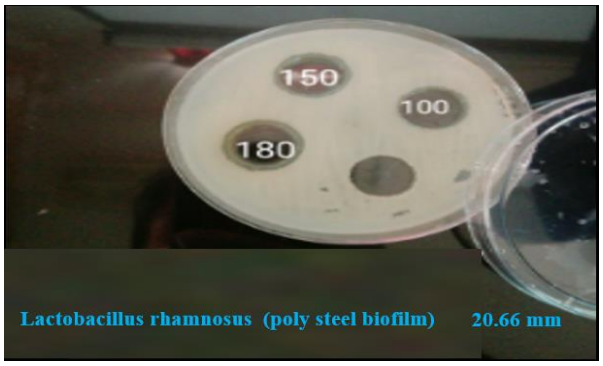


Fig. 6. Results of inoculation of *L. rhamnosus* suspension on *P. aeruginosa* biofilm at different levels

As is depicted in Figs. 7-9, the antimicrobial properties were more significant than the biofilm properties in the free bacterial state. The antimicrobial properties of the *L. rhamnosus* suspension were assessed on the surfaces of wood, glass, and polysteel, and the highest inhibition level was observed on the glass surface with a significant difference with the other surfaces in this regard (P<0.05).

Our findings demonstrated the highest antimicrobial activity against *P. aeruginosa* and its biofilm with the use of the *Glycyrrhiza glabra* extract, followed by the iron oxide nanoparticles and *L. rhamnosus* suspension (Fig. 10).

In the present study, the results of the tube dilution tests indicated that in the *P. aeruginosa* free bacteria, test tube No. 3 containing 100 mg/mL of the *Glycyrrhiza glabra* extract, test tube No. 1 containing the iron oxide nanoparticles at a concentration of 2,000 mg/mL, and test tube No. 2 containing *L. rhamnosus* at the concentration of 1,000 mg/mL were considered as the MIC and had inhibitory effects on the planktonic state of *P. aeruginosa*, while also inhibiting the growth of the bacterium without bactericidal effects (MBC).

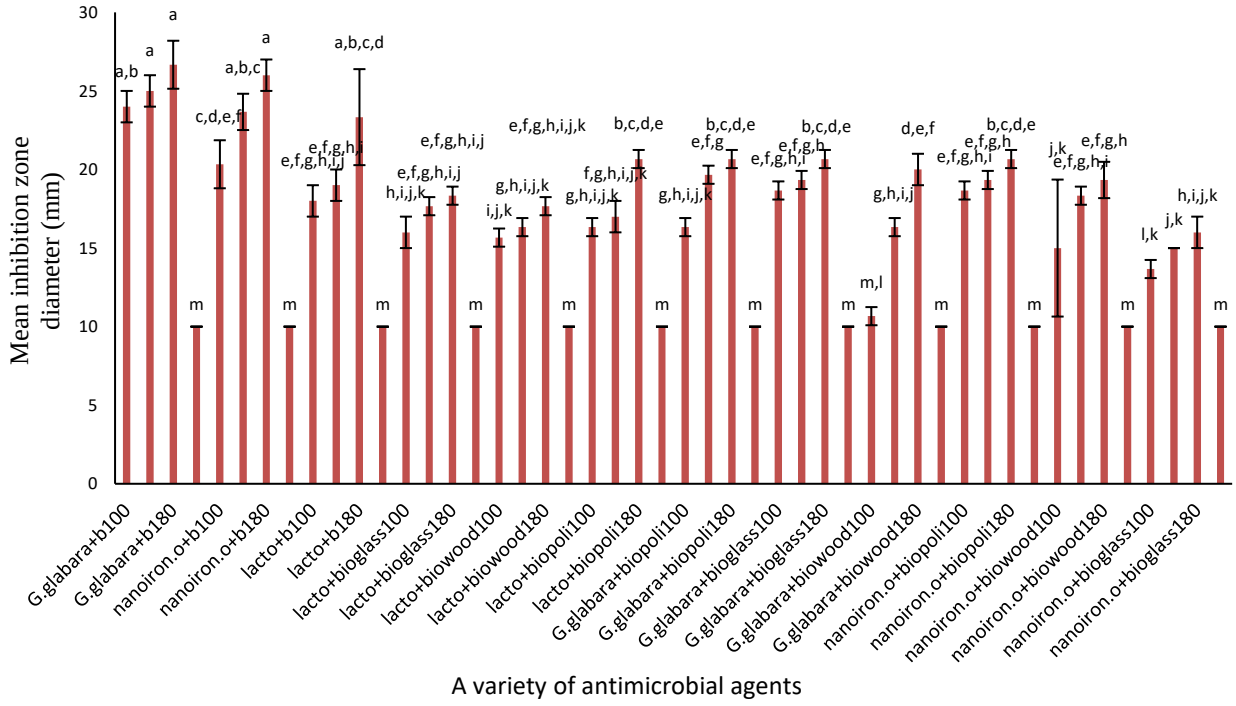


Fig. 7. Graph of mean changes of growth inhibition zone diameter of *P. aeruginosa* in different volumes (Different letters indicate significant difference between mean growth inhibition zone diameter due to antimicrobial substances; Similar letters in pattern show significant mean difference between graphs at 0.05; g, h, a: *G. glabra* + bioglass 100, h, c, d, e*: G. glabra* + bioglass 180, g, h, i; *G. glabra* + biowood 100, e, f, g: *G. glabra* + biowood 180, h, i, b, c, d: nanoiron.o + biopoli 180, f, g, h, I: nanoiron.o + biowood 100, h: nanoiron.o.o + biowood 100, j, k: nanoiron.o + bioglass 100, k: nanoiron.o + bioglass 100, a, b: *G. glabra* + b100, a, a: *G. glabra* + b 180, e, f: *G. glabra* + biopoli 180, I, k: Lacto. + biopoli 180, J, g, h: Lacto. + biowood 180, I, j, k: Lacto. + biowood 100, h, I, j, k: Lacto. + bioglass 100, h, i: Lacto. + b 180, e, f, g: Lacto. + b 100, a, b, c, a: nanoiron.o + b 180, c, d, e: nanoiron.o + b 100)

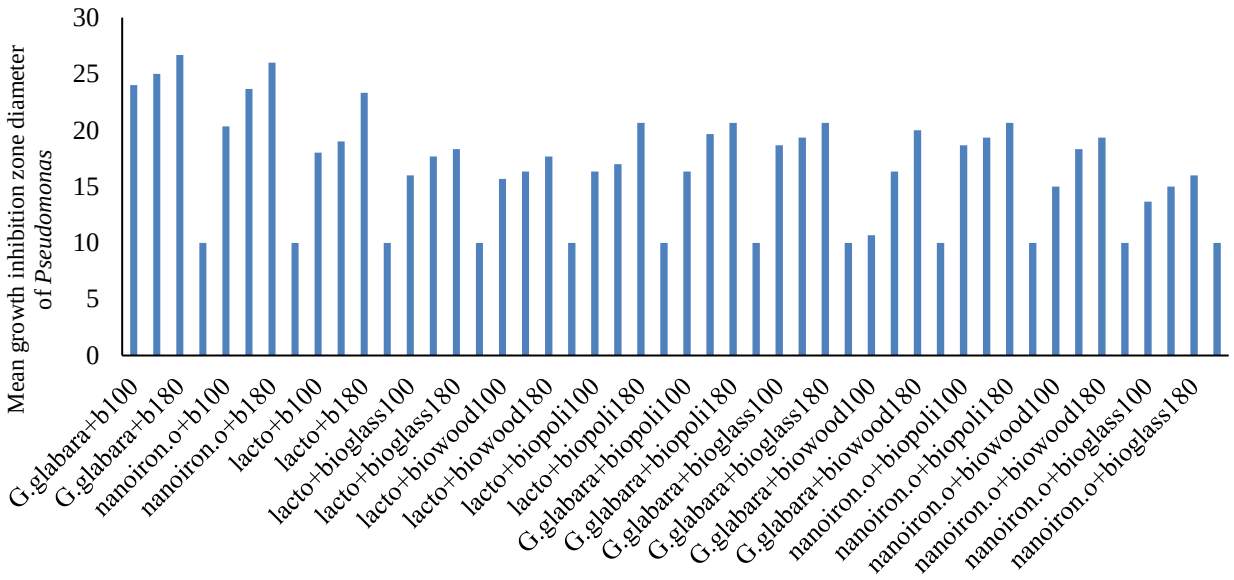


Fig. 8. Diagram of changes in mean growth inhibition zone diameter of *P. aeruginosa* biofilm against antimicrobial agents

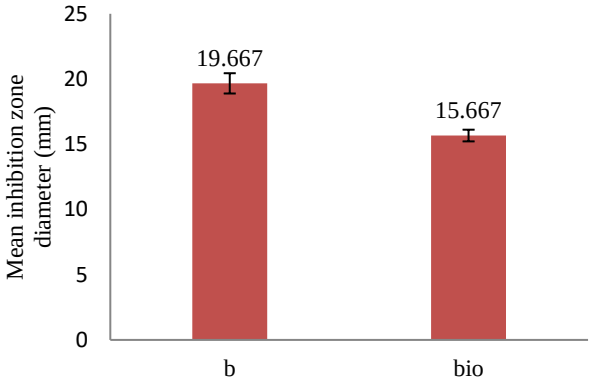


Fig. 9. General diagram of changes in mean growth inhibition zone diameter of *P. aeruginosa* in free bacterial states and bacterial biofilm

The assessment of the MIC in the biofilm of the bacterium for each of the *Glycyrrhiza glabra* antimicrobial substances on the wood, glass, and polysteel surfaces in tubes No. 4-6 showed the optimal concentration to be 100 mg/mL. As for the iron oxide nanoparticles, tubes No. 3-4-3 at the concentration of 0.2 mg/mL were considered as such, and the condition was also observed for *L. rhamnosus* in tubes No. 3, 4, and 3 at the concentration of 1,000 mg/mL. With regard to biofilm formation, each of these tubes was considered as the MIC point, indicating that each of the antimicrobial agents of *Glycyrrhiza glabra*, iron oxide nanoparticles, and *L. rhamnosus* suspension had inhibitory effects on the biofilm formation and inhibited bacterial growth with no lethal effects; therefore, no MBC point was detected in the biofilm formation.

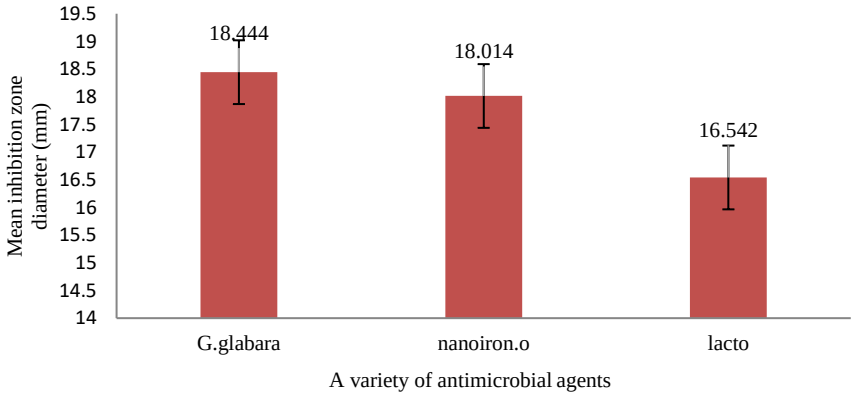


Fig. 10. Diagram of changes in mean growth inhibition zone diameter of bacterium and biofilm against different antimicrobial agents

In a study in this regard, Rahimzadeh *et al*. reported that complex probiotics (e.g., *L. rhamnosus*) have antimicrobial effects on standard *P. aeruginosa* and *P. aeruginosa* isolated from patients with burn injuries.[18] In addition, Ghotaslou and Salahi Eshlaqghi investigated *P. aeruginosa* biofilm and its new methods of prevention and treatment, reporting that various unconventional therapies have been reported across the world based on biofilm formation control techniques (e.g., surface modification and physical methods) due to the resistance of biofilms to the currently used antibiotics.[19] This is consistent with the results of the present study. In another research, Yang *et al*. discovered a new drug release system for *P. aeruginosa*, which could produce biofilms and appeared to be involved in the antibiotic resistance of biofilms.[6]

Abdi *et al*. evaluated the inhibitory effects of lactobacilli obtained from the feces of healthy neonates against the growth of *Acinetobacter baumannii* and *P. aeruginosa* with a source of nosocomial infection, reporting that due to the significant antagonistic effects of *L. plantarum* and *L. rhamnosus* against the isolates of *A. baumannii* and *P. aeruginosa*, these strains were effective in the prevention and treatment of the associated infections.[20] In another study, Ramezani *et al.* investigated the effects of iron oxide nanoparticles on a combination and non-combination with imipenem on the formation of biofilms caused by *P. aeruginosa*, concluding that the iron oxide nanoparticles, imipenem antibiotic, and their inoculation had antimicrobial effects on *P. aeruginosa*, as well as inhibitory properties, which is consistent with our findings.[21]

The domestic and foreign studies in this regard and our research indicated that the suspension of *L. rhamnosus*, iron oxide nanoparticles, and *Glycyrrhiza glabra* extract exert antimicrobial effects on *P. aeruginosa*. Therefore, biofilm formation plays a pivotal role in bacterial stability and may cause infections that are difficult to eradicate. These findings are important considering that most food contact surfaces in food processing machines are made of stainless steel.

Conclusion

According to the results, the *L. rhamnosus* suspension, *Glycyrrhiza glabra* extract, and iron oxide nanoparticles had inhibitory effects against *P. aeruginosa*. In addition, the rate of biofilm formation on the wood surface was higher compared to the other surfaces, and the greatest effect of the antimicrobial substances was observed on the polysteel and glass surfaces, respectively. Therefore, the effects of antimicrobials on biofilm were less significant, while they were more significant on the free bacteria. This is indicative of the effectiveness of the antimicrobial properties of the *Glycyrrhiza glabra* extract, iron oxide nanoparticles, and *L. rhamnosus* suspension in several medical devices.

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Hereby, we extend our gratitude to Ms. Seidi, a respected expert in microbiology laboratories, and Ms. Sohofi, a technician in the microbiology laboratory for assisting us in cultivating and passaging the bacteria and providing the necessary laboratory equipment.

Ethical issues

This Thesis is not about human beings and all ethical issues have been observed.

Credit author statement

Masoomeh Emami: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Supervision, Funding acquisition. Zahra Hojati Bonab: Methodology, Validation, Formal analysis, Writing - original draft.

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