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Research Article

Biosynthesis of titanium dioxide nanoparticles using Hypericum perforatum and Origanum vulgare extracts and their main components, hypericin and carvacrol as promising antibacterial agents

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Abstract

OBJECTIVE: To evaluate the anti-bacterial activities of titanium dioxide (TiO_2) nanoparticles of Origanum (O.) vulgare and Hypericum (H.) perforatum extracts, carvacrol and hypericin against Staphylococcus (S.) aureus.

METHODS: In this study, TiO_2 nanoparticles of O. vulgare and H. perforatum extracts, carvacrol and hypericin, were prepared and their antibacterial effects were evaluated against Staphylococcus (S.) aureus. In this study, scanning electron microscope, fourier transform infrared spectrometer, atomic force microscopy, dynamic light scattering and zeta potential were used to investigate the structure of synthesized drugs.

RESULTS: Anti-bacterial activity of synthesized NPs was tested by minimum inhibitory concentration (MIC), minimum bactericidal concentration and disc diffusion method. MICs of TiO_2-NPs synthesized using O. vulgare, H. perforatum, carvacrol and hypericin and TiO_2 were obtained 250, 62.5, 250, and 250, and 500 μg/mL, respectively. The MBCs for all of these were obtained 1000 μg/mL.

CONCLUSION: Green-synthesized of TiO_2 nanoparticles provides a promising approach to the use of O. vulgare and H. perforatum, carvacrol and hypericin as novel agents and safer antibacterial compounds, especially anti-S. aureus compounds.

Keywords: plant, medicinal; medicine, traditional; anti-infective agents; active ingredient; nano-compounds

1. INTRODUCTION

Staphylococcus (S.) species are one the most common causes of nosocomial infections in the world. [1,2] Among S. species, S. aureus has the highest pathogenicity.[3] S. aureus is one of the most important causes of community-acquired infections, which can be a major contributor to hospital infections such as bacteremial infections, endocarditis, skin infections, osteomyelitis, and toxic shock syndrome.[4] S. aureus is a gram-positive and catalase-positive bacterium and has spherical form, which usually makes irregular clusters similar to grapes. Staphylococci infections in recent years have been increasing due to the spread of resistant strains, increase in patients with immune deficiency, and excessive use of medical devices such as catheters. S. aureus is usually resistant to antibiotics such as penicillin and vancomycin.[5, 6] Today, antibiotic resistance in resistant bacteria, especially Staphylococci, is of great importance to physicians. Hence, preparation of new drugs to combat bacterial resistant is crucial. Nowadays, nanodrugs are used in natural compounds and pharmaceutical industries in order to reduce the use of chemical drugs.[7]
Titanium dioxide (TiO_2) is a metal that has many applications in the nanotechnology industry due to its

photocatalytic properties.[8] TiO_2 has several phases, crystalline, anatase, rutile and brookite, out of which anatase and rutile phases are more important due to photocatalytic properties.[9] It has low toxicity, chemical stability and low cost.[10] TiO_2 also has small size, high reactivity, chemical composition, high surface area per unit of mass.[11] For these reasons, it currently has many applications in the pharmaceutical, medical, and cosmetic industries.[12] The efficiency of TiO_2 is strongly influenced by the size, shape, and crystalline structure of nanoparticles.[13] It has antimicrobial and antibacterial properties and has no toxic effects.[14-17] Traditionally, because of the low solubility and low toxicity of synthesized TiO_2 NPs, it has been used as negative control in studies.[18] Among the different approaches to biosynthesis or green synthesis of materials, use of medicinal plants and herbal extracts is very common these days.[19] Green synthesis of herbal extracts is a safe way to reduce the risks of chemical drugs.[20] Medicinal plants and plant extracts, and certain microorganisms such as fungi and bacteria and probiotics are used as biological mediators for the synthesis and biological targets.[21-23] In this study, the plants Hypericum perforatum (H. perforatum) and Origanum vulgare (O. vulgare) and their active ingredients, i.e. carvacrol and hypericin were synthesized using TiO_2 and their antibacterial effects were investigated against S. aureus.

2. MATERIALS AND METHODS

2.1. Medicinal plants

In this study, H. perforatum (Herbarium code 48120) and O. vulgare (Herbarium code 48119) plants were collected from of Mazandaran province in northern Iran. The plants are collected and used for testing. The main active ingredients of the plants (carvacrol and hypericin) were purchased from Sigma-Aldrich and they used for the study.

2.2. Preparation of hydroalcoholic extract

The hydroalcoholic extract of the plant was prepared according to Bahmani *et al*.[21]

2.3. Synthesis of Ti O_2 NPs

For synthesis of herbs and compounds was carried out with modified method of Sanker *et al* [24] (2013). 1000 mg of hydroalcoholic of plant extracts and 30 mg of carvacrol or hypericin were added to 90 mL of TiO_2 in an Erlenmeyer flask with shaker. Each 30 min of shaking the solution, it was increased to 5 min at 50 ℃ to synthesize the materials and compounds. After 5 h of continuous stirring, the solution obtained in 50 mL falcons was centrifuged at 12 000 rpm at 4 ℃ for 15 min.

2.4. Characterization of synthesized NPs

Different techniques were used to determine the size, shape, and stability of synthesized NPs included scanning electron microscope (SEM), dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR) and zeta potential, as well as atomic force microscope (AFM).

2.5. *In vitro* antibacterial activity of synthesized NPs

The evaluation of antibacterial effect of synthesized NPs was carried out using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and disc diffusion method.

MIC and MBC were determined using the broth microdilution method proposed by the clinical and Laboratory Standards Institute (CLSI).[25] First, a stock solution was prepared from TiO_2 NPs synthesized using H. perforatum (5.000 ppm), O. vulgare (5.000 ppm), carvacrol (1.000 ppm), and hypericin (1.000 ppm). Then, 50 μL of sterile Mueller-Hinton broth was added to all wells. Next, 50 μL of stock solutions of NPs were added to the first and second rows and dilution were performed on the second to the tenth rows. Finally, 50 μL of the 24 h *S. aureus* (ATCC 12600) culture, equivalent to 0.5 McFarland turbidity (5×10^5 CFU/mL), was added to each of the second to the tenth wells, and the plates were incubated at 37 ℃ for 24 h. Methicillin (Sigma-Aldrich) was used as a positive control. After 24 h of incubation, 30 μL of 2,3,5-triphenyltetrazolium chloride was added to the visual index for bacterial growth.[25] Colorless wells were considered to indicate MIC.[25] The proposed CLSI protocol (2009) was also used to determine the MBC. Briefly, 5 μL of the solution of the wells (following the wells in which MIC formed) was inoculated on the plates containing Mueller-Hinton broth and placed at 37 °C for 24 h. The concentration at which no colony formed on the plate was considered to be MBC.[25]

To investigate the inhibition zone, on the Mueller-Hinton agar, bacterial culture of a suspension with 0.5 McFarland turbidity was inoculated into the medium with sterile swab and a disc of antibiotic methicillin was left on the medium. In addition, 40 μL of standard stocks of TiO_2 NPs synthesized with H. perforatum, O. vulgare, carvacrol and hypericin were added to the blank discs and left on the medium. The plates were then incubated at 37 ℃ for 24 h. The test was repeated three times and the results were expressed as mean ± standard deviation. The growth inhibition zone was measured by the culis.[25] The plates were then incubated at 37 ℃ for 24 h. The growth inhibition zone was measured by caliper. Each drug group was repeated three times.

2.6. Statistics analysis

Regarding the microbial tests such as MIC, MBC and disc diffusion method were repeated three times, the mean ± standard deviation was used for data analysis and reporting. In this study, mean ± standard deviation was used through Microsoft Office Pro Plus 2016 software (Excel).

3. RESULTS

The color of pure TiO_2 remains unchanged without the use of plant extracts, but when TiO_2 reacts with the plant extract or compound, color change occurs. After the reaction of titanium isopropoxide separately with H. perforatum, O. vulgare, hypericin, and carvacrol, their colors were changed. After the reaction of TiO_2 with H. perforatum, the color of extract was changed from dark green to light green. The reaction of TiO_2 with O. vulgare, the color changed from dark red to bright red. The reaction of TiO_2 with hypericin caused the color to change from red to pale hepatic. Also, the TiO_2 reaction with carvacrol changed the color from white to milky.

The FTIR spectra of synthesized TiO_2 NPs using different extracts and compounds were shown in Figure 1. Comparison of FT-IR spectra of synthesized TiO_2 NPs using O. vulgare and carvacrol shows high similarities. Also, this situation was observed for FT-IR spectra of synthesized TiO_2 NPs using H. perforatum and hypericin. The reasons for this phenomenon can be attributed to active compounds of these extracts. As we know, the active compounds of O. vulgare and H. perforatum are carvacrol and hypericin, respectively.

The morphology and size of nanoparticles were also investigated with SEM. The synthesized TiO_2 NPs using H. perforatum show spherical morphology with a diameter of 37.6-81.8 nm (Figure 2A). TiO_2 NPs synthesized using hypericin (Figure 2B). The synthesized TiO_2 NPs with O. vulgare extract represents spherical morphology for TiO_2 NPs with a diameter of 42.1-107 nm (Figure 2C). The morphology observed by the SEM images for the TiO_2 NPs synthesized using carvacrol (Figure 2D) and are also spherical and have diameters with the ranges of 29-52.2 nm and 42.1-60.1, respectively.

In the below, 2 μm × 2 μm images obtained by the Atomic Force Microscopy Analysis (AFM-Nano Surf, Switzerland) are provided below. The images of all samples were prepared by casting a solution of the samples on silicon (111) to create a thin layer of them. The mean roughness obtained for TiO_2 NPs synthesized using O. vulgare, H. perforatum, carvacrol and hypericin were equal to 52.33, 39.81, 55.39 and 90.26 nm, respectively. In samples of TiO_2 NPs synthesized using H. perforatum and NPs synthesized using hypericin, the

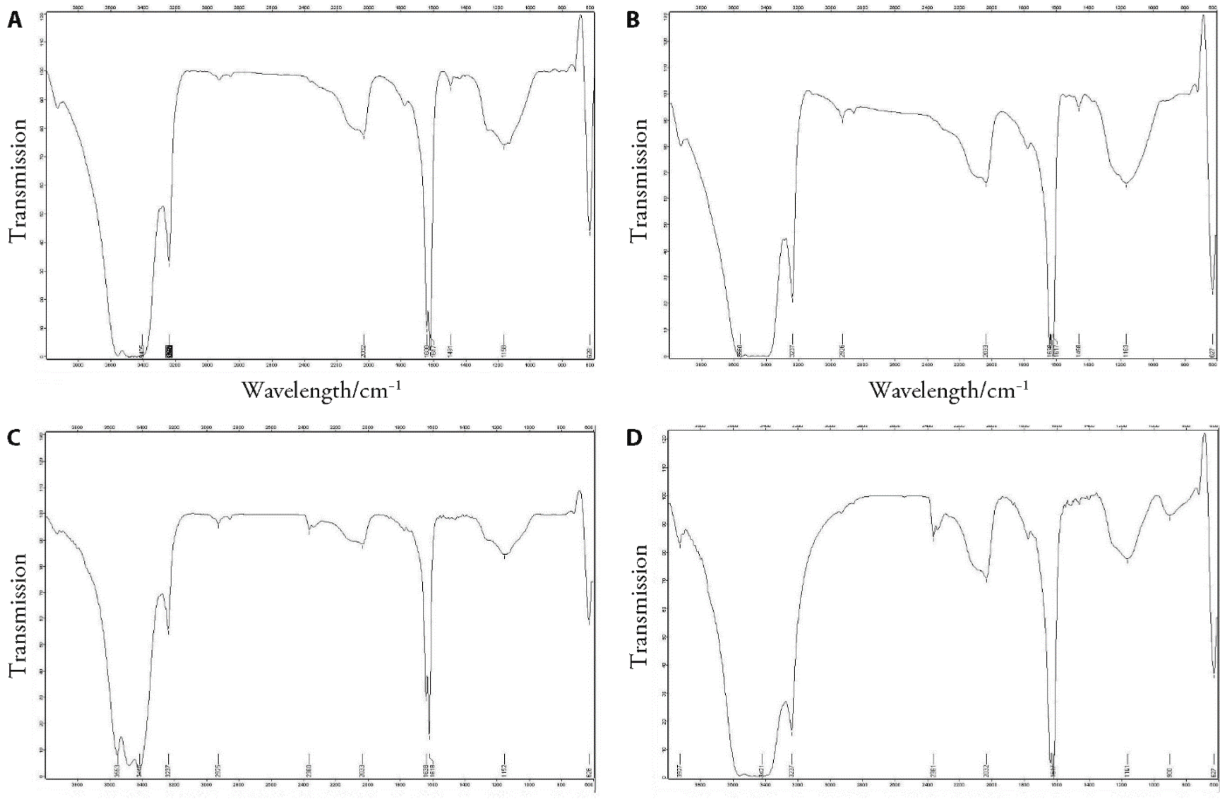

Figure 1 FTIR spectra of synthesized titanium dioxide nanoparticles

A: hydroalcoholic extract Hypericum perforatum; B: hypericin; C: hydroalcoholic extract of Origanum vulgare; D: carvacrol. FTIR: Fourier transform infrared spectrometer. Based on the analysis of the spectra of hydroalcoholic extracts of synthesezied using Origanum vulgare is observed in wavelengths of 1225 cm^{-1} (C-O stretch) and 625 cm^{-1} (C-Br stretch) of titanium bond. It is also observed for Origanum vulgare NPs. at a wavelength of 3352 O-H stretch. TiO_2 NPs. using carvacrol is observed in titanium at wavelengths of 1161 cm^{-1} (C-O stretch) and 627 cm^{-1} (C-Br stretch). It is also observed for TiO_2 NPs. synthesized using carvacrol at a wavelength of 3927 cm^{-1} (O-H stretch). TiO_2 NPs. synthesized using hydroalcoholic extract of Hypericum perforatum is also observed at a wavelength 1158 cm^{-1} (C-O stretch) and 628 cm^{-1} (C-Br stretch) are observed bond with titanium. TiO_2 NPs. synthesized using hypericin with wavelengths 1154 cm^{-1} (C-O stretch) and 626 cm^{-1} (C-Br stretch) are observed bonds with titanium.

layer growth of the samples is clearly visible on the surface, but in the sample of TiO_2 NPs synthesized using H. perforatum, island growth also occurred, while in the sample of TiO_2 NPs synthesized using hypericin, the surface is rougher, but the growth all over the surface is more uniform. In the sample of the TiO_2 NPs synthesized using O. vulgare, island growth occurred in some spots and there was a large distance between the peaks and the valleys created on surface, which made the surface of this sample less uniform than those of the other samples, while in the TiO_2 NPs synthesized using carvacrol, in all over the surface, the particles' growth was also uniform. Based on the results, the synthesis of all the nanodrugs was done well and the particle size was standard and corresponded to the particles observed in the AFM microscope (Figure 3).

The measurements of the particle size by DLS showed that TiO_2 NPs using H. perforatum (Figure 4A) 951.4 and 0.640, TiO_2 NPs using hypericin (Figure 4B) 5949 and O. vulgare had a Z-average of 1825 d.nm and pdi of 0.960 (Figure 4C). Z-average and pdi were respectively obtained for TiO2 NPs 1, and for TiO_2 NPs using carvacrol (Figure 4D) 2931and 0.640. The results showed that the particle size was well measured by the DLS.

Accordingly, synthesized TiO_2 NPs using H. perforatum extract had a potential zeta equal to +25.4 mV (Figure 5A). In addition, the zeta potential of TiO_2 NPs synthesized using hypericin, O. vulgare and carvacrol are equal to O. vulgare, carvacrol, and hypericin are equal to +5.46 mV (Figure 5B), －29.1 mV (Figure 5C) and +22.2 mV (Figure 5D), respectively. Zeta potential results show that synthesized TiO_2 NPs using H. perforatum, O. vulgare and carvacrol are stable whereas the synthesized TiO_2 NPs using hypericin has not favorable stability.

MIC of TiO_2 NPs synthesized using O. vulgare, H. perforatum, carvacrol and hypericin and TiO_2 NPs was obtained 250, 62.5, 250, and 250, and 500 μg/mL, respectively. MBC for these was obtained 1000 μg/mL MIC, MBC and zone inhibition diameter of the groups were shown in Table 1.

4. DISCUSSION

Based on the results, it was found that the highest MIC was obtained for the TiO_2 NPs synthesized using H. perforatum, followed by the TiO_2 NPs synthesized using O. vulgare, carvacrol, and hypericin. The lowest inhibitory effect was obtained for pure TiO_2 NPs. In other words, although TiO_2 NPs alone exert antibacterial effect on S. aureus, all of the herbal NPs and their compounds have lower MICs and greater antibacterial effects than TiO_2 NPs.

The results of the study of Nostro *et al* [26] on the anti-*S. aureus* effects of Origanum majorana essential oil and carvacrol, showed that the MIC of O. vulgare essential oil and carvacrol were 0.062% and 0.015% v/v and their MBCs 0.125% and 0.062% v/v, respectively. The results of this study showed that the tea of pure H. perforatum on resistant and susceptible strains of S. aureus had growth inhibition zone diameters of 13 and 12.4 mm,

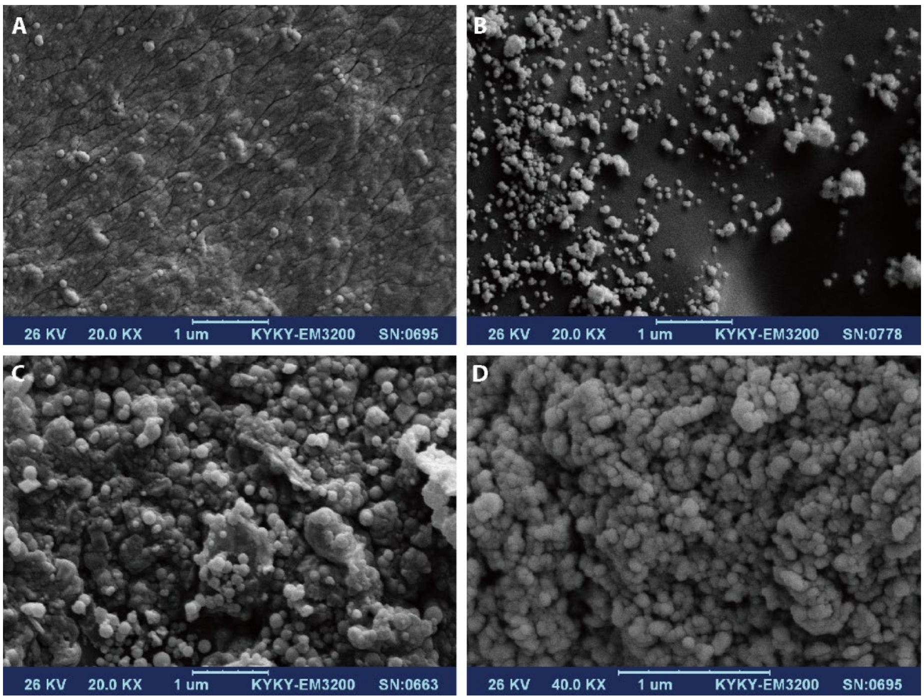

Figure 2 Scanning electron microscope images of synthesized titanium dioxide nanoparticles
A: Hypericum perforatum; B: hypericin; C: Origanum vulgare; D: carvacrol. The properties of NPs synthesized using atomic force microscope are presented in a three-dimensional visualization. The morphology of a rugged surface with the presence of both individual nanoparticles and agglomerates are described. Strong crystalline nature can be seen in diagonal figures containing mountains.

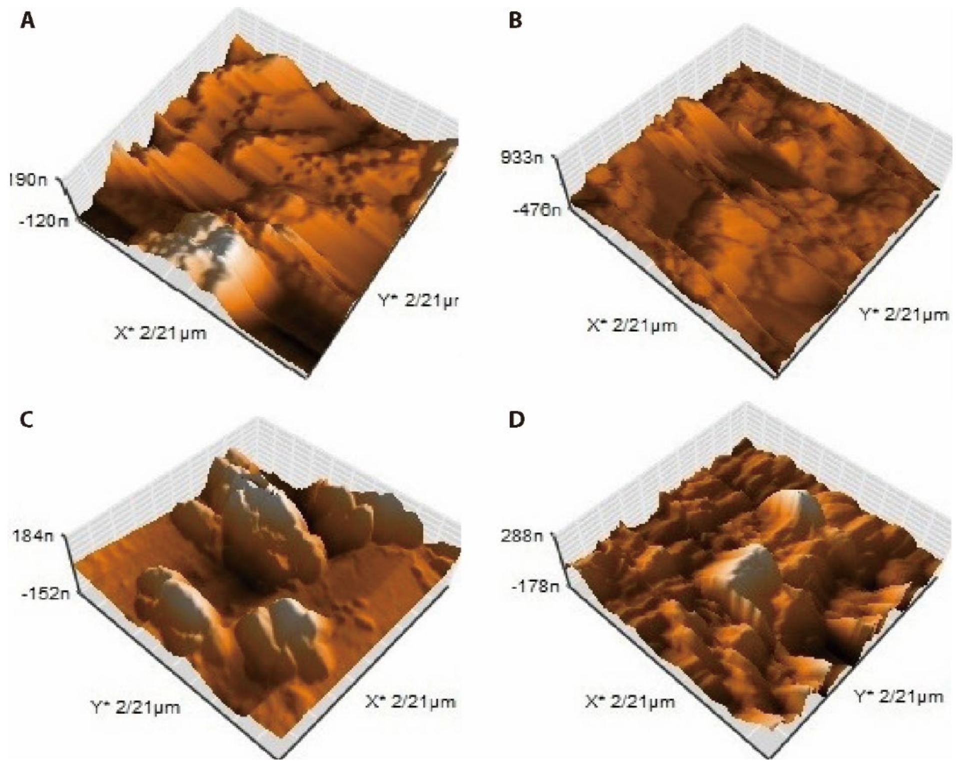

Figure 3 Atomic force microscopy images of synthesized titanium dioxide nanoparticles
A: Hypericum perforatum; B: hypericin; C: Origanum vulgare; D: carvacrol.

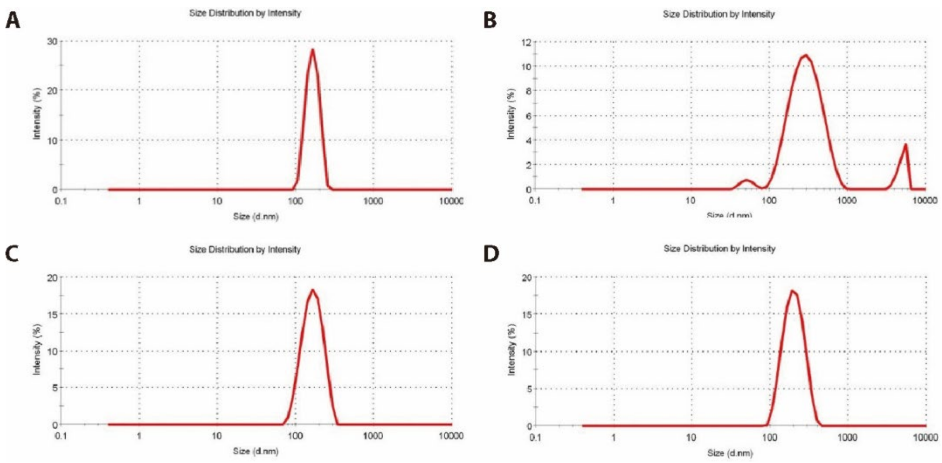

Figure 4 Dynamic light scattering analysis of titanium dioxide nanoparticles synthesized
A: Hypericum perforatum; B: hypericin; C: Origanum vulgare; D: carvacrol.

respectively.[27] The study of García *et al* [28] showed that hypericin was able to deactivate and produce antimicrobial effect on the biofilms of the methicillin resistant strains of S. aureus. In another study, hypericin-photodynamic activation in combination with acetyl-cysteine was found to have a significant potential for eradicating mature biofilms from S. aureus.[29] The results of the studies indicated the anti-S. aureus effects of the total essential oil, extract, and active ingredients, and the result of our study demonstrated the anti-staphylococcal effects of the NPs of these plants.

Our results are consistent with the cited studies. Photodynamic activity is one of the factors influencing the antimicrobial activity of plant compounds. Studies have shown that TiO_2 NPs are trapped on the surface of the bacteria, and thus the absorption of TiO_2 NPs on the surface.

In this study, the pharmaceutical agents synthesized using TiO_2 were found to produce antibacterial effects due to the presence of TiO_2 and its NPs through photocatalytic oxidation. Studies have shown that due to exposure to ultraviolet radiation or sunlight, TiO_2 has antimicrobial effects because of its strong oxidation properties. The microbe surface is the primary target of the oxidative attack, and TiO_2 particles cause contact with the microbe and produce antimicrobial effects by absorbing the radiation.[30] Since each material has its own atomic bonds, there are no two compounds with an exactly identical infrared spectrum. Therefore, infrared spectroscopy can be an efficient method to better identify

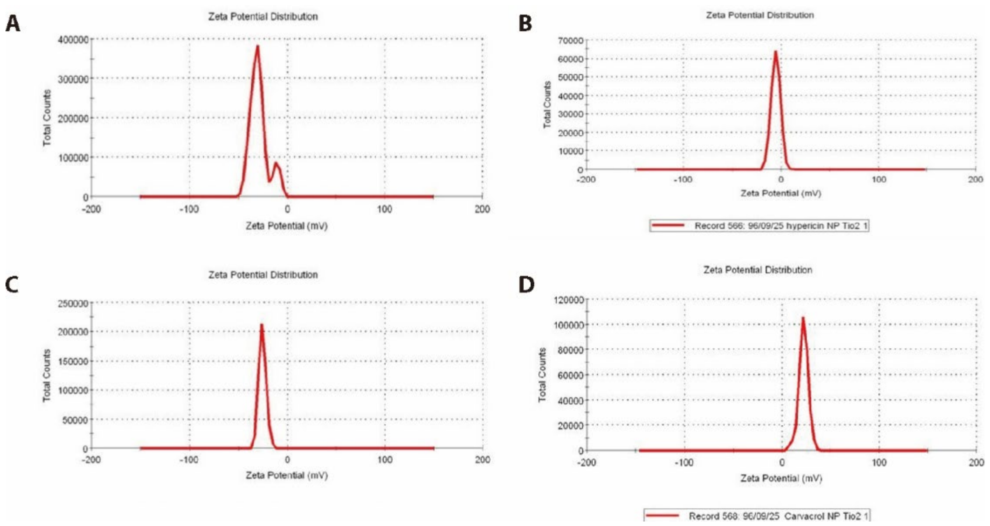

Figure 5 Zeta potential measurements of synthesized titanium dioxide nanoparticles
A: Hypericum perforatum; B: hypericin; C: Origanum vulgare; D: carvacrol.

Table 1 Minimum inhibitory concentration, minimum bactericidal concentration and zone inhibition diameter of the groups

Group	Minimum inhibitory concentration (μg/mL)	Minimum bactericidal concentration (μg/mL)	Mean ± Standard deviation (mm)
Titanium dioxide nanoparticles synthesized using Origanum vulgare	250	1000>	8.00±0.70
Titanium dioxide nanoparticles synthesized using Hypericum perforatum	62.5	1000>	7.33±0.40
Titanium dioxide nanoparticles synthesized using carvacrol	250	1000>	8.00±0.00
Titanium dioxide nanoparticles synthesized using hypericin	250	1000>	34.33±0.40
Titanium dioxide nanoparticles	500	1000>	10.00±0.00
Methicillin	384	384	22.00±0.81

different types of materials, types of functional groups, and the bonds in their molecules. Therefore, similar peaks represent the presence of the same materials.

Based on the results, the absorption peaks observed in the FTIR graphs obtained from TiO_2 NPs synthesized using H. perforatum indicated the bonds O-H, N-H, C-H, O=C=O, C=C, N-O, and S=O, which indicates the existence of the same groups in the hydroalcoholic extract of H. perforatum, which is identical to the TiO_2 NPs synthesized using hydroalcoholic H. perforatum extract. This determines that the mentioned functional groups play a role in the synthesis of NPs. In addition, the absorption peaks observed in the FTIR graph obtained from Tio_2 NPs synthesized using hypericin and other groups indicate the role of the above-mentioned functional groups in the synthesis of NPs. Functional groups reduce the bioreduction of TiO_2 particles into TiO_2 NPs. The results of the study by Huck-Pezzei *et al* [31] showed that in the FTIR test, H. perforatum had constituents at 865-3300 nm wavelengths and contained carbohydrate, nucleic acid, ether, phospholipid, lignin, etc. The results of the study by Galehassadi *et al*,[32] on the FTIR of O. vulgare, revealed that the plant had an OH phenol band at a wavelength of 3392 cm^{-1}. It also had C-H stretching bands, asymmetric stretching vibrations of aliphatic C-H, symmetric stretching vibrations of aliphatic C-H, and aromatic C=C stretching at wavelengths of 3020, 2927, 2869, and 1620/cm, respectively. Hydroalcoholic extracts of O. vulgare and H. perforatum have bioactive substances that play an important role in the synthesis of TiO_2 NPs. Based on the results obtained in this study, all the herbal NPs and their active ingredients had good antibacterial effect on TiO_2 NPs.

Metal oxide NPs exhibited different antibacterial properties depending on the surface area to volume ratio. The results of the studies show that gram-positive pathogenic bacteria exhibit greater resistance against metal NPs than Gram-negative pathogenic bacteria, which can be related to the structure of the cell wall.[33] It seems that the released TiO_2 ions in the culture medium that have positive electrical charge and cause the binding to bacterial surface proteins are the main causes of bacteriostatic and bactericidal properties of the NPs synthesized in this study. In general, the charge of the bacteria is negative and the charge of the nanoparticle positive. The same difference between the negative charge of the microorganisms and the positive charge of the nanoparticle acts as an absorbing electromagnetic load between the microbe and the nanoparticle, and causes the nanoparticle to bind to the cell surface, and thus can lead to cell death.[34] In another mechanism of action, the ions released from the NPs are responsible for reaction and binding to the thiol groups of the surface proteins of bacterial cells.[35] Besides that, NPs delay the bacterial cell adhesion and biofilm formation, which makes some groups of bacteria unable to stabilize and proliferate.[36] In addition, NPs themselves can penetrate into bacteria and cause leakage of intracellular materials out of microorganisms.[37] The ability of TiO_2 NPs to produce reactive oxygen species, and their toxicity and application[38] has attracted significant attention. Antibacterial activity of TiO_2 leads to the production of reactive oxygen species, particularly hydroxyl and peroxide free radicals under ultraviolet radiation through oxidizing and reducing pathways.[39] The causes of increased antibacterial effect of green synthesis in this study include the synergistic effects of TiO_2 NPs and O. vulgare and H. perforatum, carvacrol, and hypericin, which have antibacterial activity and produce greater antimicrobial effect by increasing the resulting surface and structure.

The study of Sankar *et al* [24] on the synthesis of aquatic O. vulgare extract synthesized using TiO_2 NPs, showed the size of the NPs was 341 nm and the negative potential 27.3 mV. In our study, hydroalcoholic O. vulgare extract, based on SEM, had a size of 42.1-107 nm, and based on the AFM test, a mean roughness of 52.33 nm and negative potential of －25.4. Our study showed that hydroalcoholic extract had a higher capacity for green TiO_2 synthesis and led to smaller NPs than the aqueous extract. Negative charge provides greater stability for TiO_2 and prevents accumulation and increase in the density of NPs.[40] In our study, TiO2 NPs synthesized using O. vulgare (－25.4 mV), H. perforatum (－29.1 mV), and carvacrol (22.1 mV), had high stability while the TiO_2 NPs synthesized using hypericin with －5.46 mV potential had a synthetically low stability. The antibacterial effect of the NPs produced against the gram-positive S. aureus showed that the MICs of the TiO_2 NPs synthesized using H. perforatum and O. vulgare, hypericin, and carvacrol were 62.5, 250, 250 and 250 μg/mL, respectively. The MBC of the produced NPs against S. aureus showed that the MBC of the TiO_2 NPs synthesized using H. perforatum and O. vulgare, hypericin, and carvacrol was all equal to 1000 μg/mL. The diameter of the growth inhibition zone of the TiO_2 NPs synthesized using H. perforatum and O. vulgare, hypericin, and carvacrol was obtained 7.3 ± 0.4, 8.0 ± 0.7, 8.0 ± 0.0, and 34.3 ± 0.4. Water-soluble heterocyclic compounds, such as flavones and ligands, reduce and absorb NPs. Functional groups associated with this cause bioreduction of $TiO(OH)_2$ into TiO_2 NPs.[41] Flavones and ligands in O. vulgare and H. perforatum are probably bioreductive agents of synthesized TiO_2 NPs, which result in a more effective antibacterial effect of green synthesis. The results of the study by Barzegar *et al* [42] showed that 1.5% TiO_2 NPs caused cell death of S. aureus after 24 h. Its mechanism of action is through the peroxidation of the cyclic phospholipid membrane and disruption of membrane permeability. The results of the study of Saadatmand *et al* [43] showed that chitosan-TiO_2 nanocomposite was nearly 100% effective on the growth of S. aureus. The results of a study showed that TiO_2 NPs in combination with β-lactam, cephalosporins, amin-oglycosides, glycopeptides, macrolides, lincosamides, and tetracycline were able to exhibit antimicrobial activity against methicillin-resistant S. aureus.[44] Antimicrobial properties of medicinal plants are usually due to phenolic compounds, saponins, and flavonoids in their structure. These commonly used ingredients usually affect the permeability of the cytoplasmic membrane and its structural enzymes and exert their medicinal effect. Therapeutic and medicinal effects of herbal and aromatic plants through their active ingredients and antioxidants.[45,46] It is essential to find nanodrugs that are synthesized from medicinal plants and used as medicine.[47-54] Effective compounds and herbal antioxidants can be used as effective drugs for medicinal purposes.[55-62] Based on the results of this study, we can conclude that TiO_2 NPs (synthesized using O. vulgare, H. perforatum, carvacrol, and hypericin) alone have anti-S. aureus properties. The physicochemical properties of their NPs were confirmed by the use of the DLS, SEM, FTIR, AFM, and Zeta potential. All four synthesized TiO_2 NPs had optimal antimicrobial effects against S. aureus. The results provide conclusive evidence for the antibiotic activity of synthesized TiO_2 NPs. The authors are confident that in the near future, green-synthesized TiO_2 NPs can be used as a new and effective antibiotic and therapeutic agent for the treatment of infectious diseases. Green synthesized provide a promising approach to the use of extracts of O. vulgare and H. perforatum, and carvacrol and hypericin to fulfill the industrial need for antimicrobial compounds, especially anti-S. aureus agents, that are also new, simplified, low cost, environmentally friendly, and recyclable.

5. ACKNOWLEDGMENT

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