Journal of Computational Applied Mechanics 2022, 53(1): 41-54

DOI: 10.22059/jcamech.2021.336523.684

RESEARCH PAPER

The influence of hydro alcoholic extract of Satureja mutica zinc Oxide nanoparticle and zinc complex against coagulase gene expression on standard and clinical isolated of methicillin resistant staphylococcus aureus

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most important hospital pathogens that cause of severe morbidity and mortality worldwide. Coagulase gene (Coa) is a pathogenicity factor of this bacterium which found in all pathogenic strains. The aim of this study was to evaluate the antibacterial effect of hydroalcoholic extract of Satureja mutica, zinc oxide nanoparticle and zinc complex alone and simultaneously on inhibiting the growth of clinical and standard of coagulase gene expression isolates of MRSA. Minimum Inhibitory Concentration (MIC), cell viability and zone of inhibition were determained by microdilution method. MTT assay and disk diffusion method were performed against MRSA strains that possess coa gene respectively. In addition, the changes in expression of this gene were investigated by RT-PCR in MIC concentration. Hydroalcoholic extract of Satureja mutica, zinc oxide nanoparticle, and copper nano-complex showed antibacterial and inhibitory effects on MRSA growth. Additionally, a significant inhibitory effect was seen on coagulase gene expression in presence of hydroalcoholic extract of Satureja mutica. Frurthermore, the MIC for clinical and standard MRSA were determined as 1500 and 3000 µg/ml for the Satureja mutica extract, 20 and 40 µg/ml for the zinc oxide nanoparticle, and 20 and 40 µg/ml for the Zinc complex respectively. After performing RT-PCR, the results of electrophoresis showed that Satureja mutica extract in MIC induced an inhibitory effect on coagulase gene expression. Using Satureja mutica extract with nanoparticles in MIC concentration showed a synergistic effect in coa gene expression. However, no effect was observed on housekeeping arcC gene. Zinc oxide nanoparticles also have inhibitory effects on the growth of bacteria but no inhibitory effect was observed on expression coa gene.

Keywords: MRSA, CuO, Cu complex, coagulase gene, Satureja mutica

1. Introduction

Since bacterial resistance to antibiotics is increasingly on the rise, medical science researchers seek to replace

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these antibiotics. Nanotechnology is the branch of technology that deals with dimensions and tolerances of less than 100 nanometers, especially the manipulation of individual atoms and molecules. Since the discovery of nanotechnology by Feynman [1-8]. It is noteworthy that the use of medicinal plants and nanoparticles can play an important role to replace these antibiotics. Among medicinal plants, 14 species savory (*Satureja*) from mint family (*Lamiaceae*) have been reported in Iran [9]. The main components of the extract obtained from some species such as Polgen, menthol carvacrol and terpinene. While, Seaman constitute is a major component of the extract in some other species. Obviously, depending on the type and percentage of components, extract application is also different [10]. The main components of the extract obtained from some species such as Polgen and menthol, while, carvacrol, terpinene, and Paralympic. Seaman constitute is a major component of the extract in some other species. Obviously, depending on the type and percentage of components, extract application is also different [10]. Different species of a savory genus have a lot of variety in terms of the extracted amount and chemical compositions. Subsequently, metal nanoparticles are considered by scientists due to their different optical, chemical, electrical and photoelectrochemical properties.

Different species of a savory genus have a lot of variety in terms of the extracted amount and chemical compositions [11, 12]. Since the metal nanoparticles are considered by the scientists due to the different optical, chemical, electrical and photoelectrochemical properties [13]. It is known that many heavy metals in very small concentrations kill the bacteria. The main mechanism of the effect of nanoparticles on bacteria is the damage to DNA, proteins and cell wall destruction [14]. Gram-positive bacteria are more resistant to metal nanoparticles compared to gram-negative bacteria and this could be related to the structure of the cell wall [15]. Among the nanoparticles, zinc oxide and zinc complex have been used. Zinc oxide nanoparticles have numerous applications in the pharmaceutical and medical aspects and are commonly used as sunscreens and antibacterials (Prach, Stone, and Proudfoot, 2013). The combined affinity of zinc oxide and bacterial cells is an important factor for antibacterial activity. The main mechanism of toxicity of zinc oxide nanoparticles is the dissolution and release of zinc ions. Zinc nanoparticles are optionally toxic and act as harmless reagents for human cells (Matai *et al*., 2014). MRSA is a major cause of bacterial infections including bacteremia, lower respiratory tract infections, skin and soft tissue infections, and is the most common cause of nosocomial infections among people with the most immunodeficiency [16]. Due to the high genetic diversity of Staph aureus and the ability to alter antibiotic susceptibility, most S. aureus clinical isolates are resistant to some antibiotics [17]. Zinc nanoparticles have optional toxicity and act as a harmless reagent to human cells[16]. *MRSA* is the main cause of bacterial infections including bacteremia, lower respiratory tract infections, skin and soft tissue infections, and the most common cause of *nosocomial infection* among people who are mostly immunocompromised [18]. Because of the high genetic diversity of the *Staph aureus* and the ability to change the sensitivity to antibiotics, most clinical isolates of *S. aureus* are resistant to some antibiotics [17].

S. aureus due to the presence of the mecA gene, which encodes the penicillin-binding protein (PBP2a), has a lower affinity for the beta-lactam ring in these antibiotics, leading to bacterial resistance to beta-lactam antibiotics [19]. All pathogenic MRSAs have the pathogen coagulase gene, and the position of this gene is located between the lipase gene and protein A which is controlled by the lateral gene regulatory system (Agr) [20]. The aim of this study was to evaluate the effect of hydroalcoholic extract of Satureja mutica, zinc nano oxide and zinc complex simultaneously on inhibiting the growth of clinical isolates and MRSA standard and also the effect of each of these substances alone on coagulase gene. The collected data were analyzed by SPSS software.

S.aureus is resistant to methicillin due to the presence of the *mecA gene* which encodes a *modified penicillin-binding protein (PBP2a*) that enjoys a lower affinity for beta-lactam ring in these antibiotics and leads to resistance of bacteria to beta-lactam antibiotics [19]. All disease-causing *MRSAs* have pathogenic *coagulase* gene and the position of this gene is on the *MRSA* genome between *lipase gene* and protein A and is controlled by the accessory gene regulatory system (*Agr*) [20].

The aim of this study is evaluating the effect of *Satureja mu*tica hydroalcoholic extract, zinc nano-oxide and zinc complex alone and simultaneously on inhibiting the growth of clinical and standard isolates of *MRSA* as well as the impact of each of these substances alone on *coagulase* gene expression in the mentioned bacterial samples. Moreover, the collected data were analysed with SPSS software.

2. Materials and Methods

2.1. Preparation of Extraction

Satureja mutica was collected in late summer (August) during the flowering stage from the heights of Rudsar city (Eshkorat region) in Gilan province. The hydroalcoholic extract was prepared after drying in the shade using the liquefaction method. Two hundred grams of Satureja mutica powder was placed in 1000 ml of hydroalcoholic

extract (500 ml of 96% ethanol with 500 ml of distilled water) and placed on a shaker for 48 hours. The solution was filtered through Whatman filter paper No. 1 and the solvent was removed from the environment using a rotary apparatus (Hydolph, type: Heizbad hei-VAP, Germany) and the extract was poured into a sterile plate and stored at 4 ° C (Askarinia, M., & etal).

The shoot off (forest savory) *medicinal* plant namely *Satureja mutica* was collected during flowering stage from the highlands of Roodsar city (*Eshkevarat* Area) in Gilan province in late summer (August). The hydroalcoholic extract was prepared from it after drying in the shade using maceration method. Two hundred grams of *Satureja mutica* powder was placed in 1000 mL of *hydroalcoholic extract* (500 ml ethanol 96% with 500 ml distilled water) and put on shaker for 48 hours. The solution was filtered by *Whatman filter paper No. 1* and the solvent was removed from the environment using Rotary machine (Hydolph, type: Heizbad hei-VAP, Germany) and the obtained extract was poured in sterile plate and stored at 4 ° C [21].

2.2. Preparation of nanoparticles

Synthesis of Ligand 1 (L1):

L1 = (pyridin-2-ylmethylene) Isonicotinic hydrazide (Hp-2-minh)

Isonicotinohydrazide (1.37 g, 10 mmol) was added to a solution of picolinaldehyde (2.20ml, 20 mmol) in methanol (100 mL) and the mixture was refluxed for 24 h. A white solid formed which was filtered off, washed with methanol and vacuum dried (Yield: 91%). M.p. 157 °C.

IR υ_{max}; 3448, 3047, 2923, 1663, 1542, 1461, 1400, 1273,1143,1060, 846, 874, 682 Cm^{-1} (KBr).

^{1}HNMR ($CDCl_3$, ppm): 7.79 (d,2H), 8.71 (d,2H), 7.916 (t,1H), 7.40 (t,1H), 7.45 (d,1H), 7.55 (s,1H).

Nanostructure of $[Zn(L1)(Br)_2]$, 15 mL of a 0.1 M solution of Zn(II) Bromide in H_2O was positioned in a high-density ultrasonic probe, operating at 20 kHz with a maximum power output of 600 W. Into this solution, 15 mL of 0.2 M solution of the ligand L1, were added dropwise. The obtained precipitates were filtered off, washed with water and then dried in air.

Product 1: d.p.= 239°C. Found; C: 32.00, H: 3.00, N: 12.10%. calcd. for $C_{12}H_{10}Br_2N_4OZn$: C: 31.93, H: 2.23, N: 12.41 %.

IR selected bonds: ν= 3157*s*, 3025*w*, 1650*s*, 1530*s*, 1467*s*, 1158*m*, 1084*m*, 754*w*, 694*w*, 637*w*.

To isolate single crystals of $[Zn(L1)(Br)_2]$, ligand L1 (0.30 g, 1 mmol) was placed in one arm of a branched tube and Zn(II) Bromide (0.23 g, 1 mmol) in the other. Methanol was then carefully added to fill both arms, the tube was sealed and the ligand-containing arm immersed in a bath at 60°C, while the other was left at ambient temperature. After 3 days, crystals suitable had been deposited in the arm at ambient temperature. They were then filtered off, washed with acetone and ether, and air dried. Yield: 70%.

Product 1: d.p.= 240°C. Found; C: 32.00, H: 2.30, N: 12.10%. calcd. for $C_{12}H_{10}Br_2N_4OZn$: C: 31.93, H: 2.23, N: 12.41 %.

IR selected bonds: ν= 3158*s*, 3026*w*, 1650*s*, 1530*s*, 1467*s*, 1158*m*, 1084*m*, 754*w*, 694*w*, 635*w*.

2.2. Collecting strains under study

In this study, the clinical samples were isolated from *patients* hospitalized in *Yasuj Shahid Beheshti Hospital* and identified using standard microbiology regarding to CLSI. The *MRSA standard co1 strain was purchased from the Pasteur Institute of Iran (IPI)* and was subject to all experiments along with the clinical samples. Due to PCR reaction, the presence of *coagulase gene* was confirmed in all strains under study.

2.3. Determine the Minimum Inhibitory Concentration (MIC) of nanoparticle, nano complex and extract:

To determine the MIC, the broth microdilution method was used according to CLSI standards.

In this test, the concentrations of 5, 10, 20, 40, 80 and 160 µg/ml were used for zinc oxide nanoparticle, zinc complex and the concentrations of 375, 750, 1500, 3000, 6000 and 12000 µg/ml of *Satureja mutica* extract were tested. Moreover, to determine the simultaneous effect of zinc oxide nanoparticle, zinc complex, and *Satureja mutica* extract, different concentrations of zinc oxide nanoparticle and zinc complex with *Satureja mutica* extract were created in the wells. Accordingly, the created concentration in the first well was equal to that of MIC from each of the substances and other concentrations were obtained from dilution. Finally, 10 µl of bacterial suspensions that prepared was equal to Half McFarland tube (5×10^6 CFU / ml) added to all wells while the final volume per well was 100 µl.

A row of wells was considered to confirm the bacterial growth as a positive control containing only bacteria and bacterial growth medium and a row was considered as the blank which included study materials and the culture medium. Microplate was incubated for 20 hours in a shaking incubator at 37 °C and the results were read through

the eyes (observation of turbidity caused by the growth of bacteria) and optical density (OD) of plates were read by ELISA plate reader at a wavelength of 620 nm and the percentage of inhibition was measured with Formula (1). To ensure each test was repeated three times [22, 23].

$$\text{Percentage of inhibition} = \left(1 - \frac{a}{b}\right) \times 100 \quad (1)$$

a: absorbance of treated wells
b: absorbance of control

2.4. MTT determination

This test is based on rehabilitating and leaching yellow crystals of tetrazolium (MTT) and converting it to blue and insoluble Formazan crystals in Bacterial cytoplasm. These crystals are turned into solution by a solvent such as DMSO.

MTT is determined according to the MIC technique so that after 20 hours and determining the MIC, 5µl of *MTT solution* (*5 mg/ml) is added* to all wells of the mentioned plates and incubated for 1 hour in the dark at 37 ° C. Then 100 mL of DMSO was added to the wells and incubated for 3 hours at 37 ° C. The MTT colour change from yellow to purple indicated the presence of live bacteria in the wells and the absence of live bacteria in the wells (when there was no colour change). Furthermore the lowest concentration of materials in which no colour change was observed was considered as MIC. Then the optical density (OD) of plates was measured at a wavelength of 570, and the viability rate of wells was determined according to Formula 2 [23, 24].

$$\text{Percentage of inhibition} = \left(\frac{a}{b}\right) \times 100 \quad (2)$$

a : absorbance of treated wells
b : absorbance of control

2.5. The determination of zone of inhibition diameter

To determine the zone of inhibition, the Kirby Bauer method was used in bacterial suspension equal to Half McFarland was prepared and *cultured* on Mueller Hinton agar medium (Pure plate method) using a sterile swab. Then 25µl of the *Satureja mutica* extract, zinc oxide nanoparticles, zinc complex, *Satureja mutica* extract with zinc oxide nanoparticles, and *Satureja mutica* extract with zinc complex in the concentration of MIC were added to a blank disk and placed on the culture medium and incubated separately at 37 ° C for 24 hours. A disc containing 25µl of DMSO was added on each plate as a blank. The tests were repeated three times for each bacterium [25].

2.6. Antibiogram

To determine the sensitivity of MRSA bacteria under study, a suspension of half McFarland was prepared from bacteria and *cultured* on Mueller Hinton agar medium. In the following, *antibiotic vancomycin* discs (30µg), Erythromycin (15µg), penicillin (10µg), gentamicin (10µg) and ciprofloxacin (5µg) were putted on the plates and incubated at 37 ° C for 24 hours. According to zine of inhibition diameter and based on CLSI standards, sensitivity and resistance of bacterial strains to these antibiotics were determined.

2.7. PCR to determine coa gene

The presence of *coa* gene was confirmed with the *Sinnagen company kit* using PCR molecular method. The reaction mixture is used for *coa gene* PCR to a final volume of 25 µl as follows:

Master Mix (1x): 12.5 µl, Primer F (0.1 - 1µM) 1µ, Primer R (0.1 - 1µM): 1µ, Template DNA: 5µ (20pg), Sterile Deionized Water: 5.5µ,

PCR stages for the detection of *coa gene* was performed according to the following procedure:

Initial denaturation: 95°C, 120s, 1cycle, denaturation: 93°C, 45s, 34cycle, Annealing: 54°C, 60s, 34cycle, Extension: 72°C, 30s, 34cycle, Final Extension: 72°C, 90s, 1cycle.

2.8. Gene Expression

Gene-specific primers were designed with Genscript software for *coa gene* and *housekeeping gene carbamate kinase* (*arcC*) as internal control (Table1). Then the strains under study were treated with nanoparticles and the extract was held on concentration of MIC for 24 hours at 37°C in Mueller Hinton Broth and also cultured as the positive control strains without materials under study. After preparation of *precipitation, RNA* was extracted according to Kit Cinnagen guidelines and converted to cDNA. Moreover, the level of *coa* and *arcC genes* expression were determined by RT-PCR method as follows:

Coa gene expression: Initial denaturation: 95°C, 120s, 1cycle, denaturation: 93°C, 45s, 34cycle, Annealing: 54°C, 60s, 34cycle, Extension: 72°C, 30s, 34cycle, Final extension: 72°C, 90s, 1cycle

arcC gene expression: Initial denaturation: 95°C, 120s, 1cycle, denaturation: 93°C, 45s, 34cycle, Annealing: 56°C, 60s, 34cycle, Extension: 72°C, 30s, 34cycle, Final extension: 72°C, 90s, 1cycle.

Table1. Primer sequences and annealing temperature

gene	sequence of primers	annealing temperature (°C)	Number of cycles	length of the chain (BP)
coa	F: CATACAAGAAGCCAAGCGAA R: ACTTGACCGTTTGCATGTGT	54	34	142
arcC	F: TTGATTCACCAGCGCGTATTGTC R: AGGTATCTGCTTCAATCAGCG	56	34	570

2.9. Statistical Analysis

The MIC and ZOI of the study's treatments against MRSA reported in Tables 2 and 3 respectively. According to the result of the Kolmogorov–Smirnov test, these mentioned variables had not a normal distribution ($p=0.001$), so the result of non-parametric tests such as the Sign test and Kruskal–Wallis reported in tables 2 and 3.

As shown in Table 2, the MIC of Zinc Nano complex was less than the MIC of Zinc Oxide Nanoparticle and *Satureja Mutica* Fisch Extract. Also, the MIC of Extract, Nanoparticle, and Nano complex in the synergies status were less than the MIC of the use of each of them alone. The result of Kruskal–Wallis test, and Scheffe procedure as Post Hoc multiple comparisons confirmed that according to the MIC of the study's treatments, Zinc Nano complex has a meaningful stronger antibacterial effect against Methicillin-Resistant Staphylococcus Aureus (MRSA) in comparison with Zinc Oxide Nanoparticle and *Satureja mutica* Fisch Extract, and likewise, Zinc Oxide Nanoparticle has a meaningful stronger antibacterial effect than *Satureja mutica* Fisch Extract against MRSA ($p<0.05$). Also, the Synergism of Extract, Nanoparticle, and Nano complex causes a great increase in the antibacterial effect of them against MRSA ($p<0.05$).

Table 3 indicates that the ZOI of MRSA in the presence of *Satureja mutica* Fisch Extract was more than the ZOI of MRSA in the presence of Zinc Nano complex and Zinc Oxide Nanoparticle. In addition, the ZOI of MRSA in the presence of Zinc Nano complex was more than the ZOI of MRSA in the presence of Zinc Oxide Nanoparticle. Also, the ZOI of MRSA in the presence of Extract, Nanoparticle, and Nano complex in the synergies status were more than the ZOI of MRSA in the presence of each of them alone. The result of Kruskal–Wallis test, and Scheffe procedure as Post Hoc multiple comparisons confirmed that according to the ZOI of MRSA in the presence of the study's treatments, *Satureja mutica* Fisch Extract has a meaningful stronger antibacterial effect against Methicillin-Resistant Staphylococcus Aureus (MRSA) in comparison with Zinc Nano complex and Zinc Oxide Nanoparticle, and likewise, Zinc Nano complex has a meaningful stronger antibacterial effect than Zinc Oxide Nanoparticle against MRSA ($p<0.05$). Also, the Synergism of Extract, Nanoparticle, and Nano complex causes a great increase in the antibacterial effect of them against Methicillin-Resistant Staphylococcus Aureus ($p<0.05$).

It must be acknowledged that according to the result of the Sign test that reported in tables2 and 3, the MIC of the study's treatments against the clinical isolate of Methicillin-Resistant Staphylococcus Aureus and the ZOI of the clinical isolates of MRSA in the presence of all study's treatments have meaningful differences with these indicators of the standard strain of MRSA ($P<0.05$).

3. Results

In this study, 15 MRSA strains consisting of a standard strain and 14 clinical strains isolated from burn wounds of hospitalized patients that were confirmed by specific diagnostic tests were used in this study.
In this study, 15 strains of MRSA, consisting of a standard strain and 14 clinical strains isolated from burn wound from hospitalized patients and confirmed with specific diagnostic tests of this bacterium were treated and applied for the study.

3.1. Characterization of nanoparticles

The acceptable match, with slight differences in peak positions was observed between the simulated and experimental powder. X-ray diffraction patterns indicate that the nano-structure sample is a single crystalline phase identical to that obtained by single crystal diffraction. The significant broadening of the peaks of the nanostructure indicates that the particles are nanometer dimensions. The average size of the particles was estimated by the Sherrer formula, $D = 0.891\lambda/\beta\cos\Theta$, where D is the average grain size, λ the X-ray wavelength (0.15405 nm), Θ the diffraction angle and β the full-width at half maximum of an observed peak. The obtained value was D = 32 nm.

Fig.1 shows the nano-layers were observed by scanning electron microscopy. The morphology of compound **1** prepared by the sonochemical method.

The powder XRD patterns (Fig. 2), which match the standard pattern of hexagonal ZnO with *P63mc* space group (JCPDS card file No. 36-1451), confirmed the formation of ZnO powder. The significant broadening of the peaks of the nanostructure indicates that the particles are nanometer dimensions. The average size of the particles was estimated by the Scherrer formula. The obtained value was D = 44 nm.The morphology and size of the prepared ZnO samples were further investigated using SEM. The [Zn (L1) $(Br)_2$] nanostructure produced regularly shaped Zn (II) oxide nanoparticles with a diameter of about 44 nm.

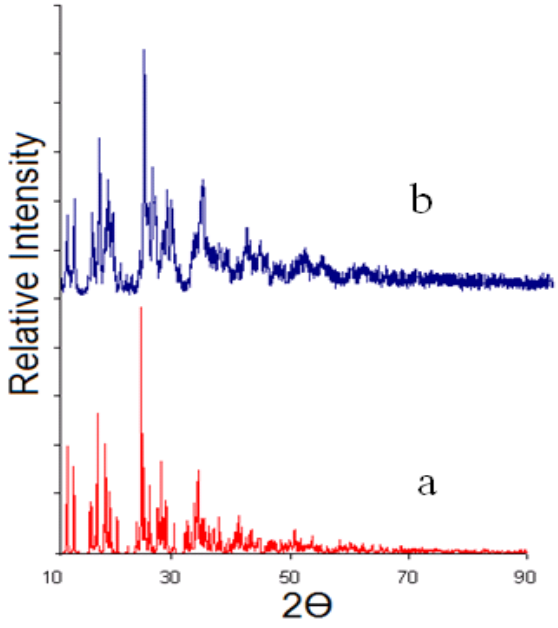

Fig. 1. The XRPD patterns of (a) computed from single crystal X-ray data of compound 1; (b) nano-structure of compound 1.

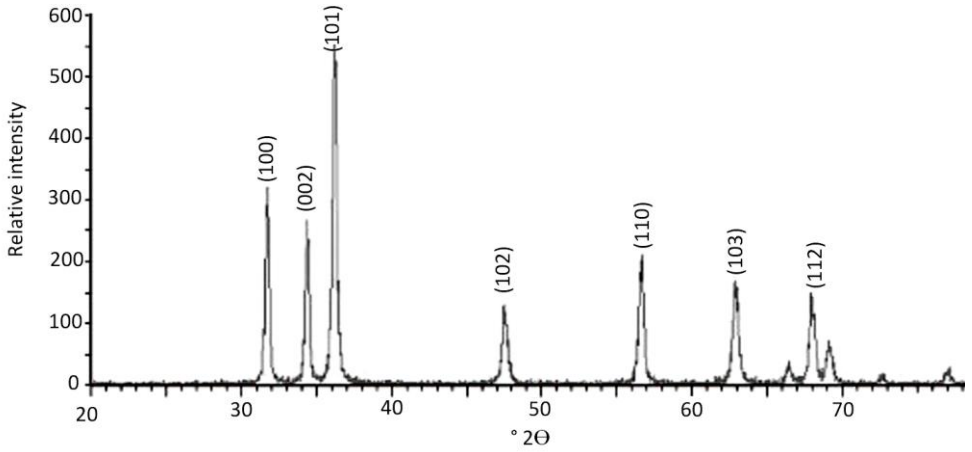

Fig. 2. XRD pattern of ZnO after the calcination of 1.

3.2. Determining the minimum inhibitory concentration (MIC) and inhibition zone

The minimum inhibitory concentration and inhibition zone diameter in the treatments strains of *MRSA* are presented in Tables 2 & 3. Since these variables lacked of normal distribution according to Kolmogorov – Smirnov test (P=0.0001). The results of non-parametric tests such as Sign test and Kruskal-Wallis test have been reported in these tables. According to table 2, the MIC of zinc compex is less than zinc oxide and *Satureja mutica* extract, and the MIC of the zinc oxide nanoparticle is also less than *Satureja mutica* extract. Besides, the MIC of simultaneous use of *Satureja mutica* extract with zinc oxide nanoparticle or zinc complex is less than each of the mentioned treatments alone. Kruskal-Wallis test and Scheffe post hoc test confirmed ~~at~~ the high statistical significance level that zinc nanocomplex was lower than zinc nanoparticle and *Satureja mutica* extract according to the MIC indicator results and the simultaneous use of the two extracts and the nanoparticle or extract and that nanocomplex also lead to a drastic reduction in MIC concentration of the mentioned treatments against MRSA ($P<0.05$).

According to table 3, *MRSA inhibition zone diameter* in the presence of *Satureja mutica* extract is more than *bacterium inhibition zone diameter* in the presence of zinc nanocomplex and zinc oxide nanoparticle. *MRSA inhibition zone diameter* in the presence of zinc noncomplex is more than this indicator in the presence of zinc oxide nanoparticle. Besides, the *MRSA inhibition zone diameter* in simultaneous use of *Satureja mutica* extract with zinc nanocomplex and zinc nanoparticle was more than *bacterium inhibition zone diameter in the presence of each of the mentioned treatments alone. Kruskal-Wallis test and Scheffe post hoc test confirmed at the statistically significant level. According to the inhibition zone diameter indicator results, Satureja mutica extract enjoys more inhibition zone diameter on MRSA than zinc nanocomplex and zinc nanoparticle. Besides, zinc nanocomplex has an inhibition zone diameter of zinc nanoparticle on MRSA and the* simultaneous *use of this extract and the nanoparticle or extract and nanocomplex, leads to a significant increase in inhibition zone diameter of the mentioned treatments against MRSA (P<0.05).*

3.3. Antibiotic susceptibility

The MRSA *inhibition zone diameter* was compared with the presence of the study treatments with bacterium in the presence of the selected antibiotics available in clinical and laboratory standards institute (CLSI) including Vancomycin, Erythromycin, Penicillin, Gentamycine, Ciprofloxacin. Accordingly, the antibacterial power of treatments under study against MRSA was determined. A *zone of inhibition diameter* of selected antibiotics compared with standard and clinical MRSA strains under study with *a zone of inhibition diameter of all strains tested with Satureja mutica extract, zinc oxide nanocomplex and zinc complex and the simultaneous use of Satureja mutica extract with the zinc nanocomplex and simultaneous use of Satureja mutica extract with the zinc nanoparticle.*

3.4. Determining the viability of bacteria using MTT assay

The viability rate of the standard and clinical strains of *MRSA* at the presence of different concentrations of *Satureja mutica* extract, zinc oxide nanoparticles, zinc nanocomplex, simultaneous use of *Satureja mutica* extract with zinc nanoparticle and the simultaneous use of *Satureja mutica* extract with zinc nanocomplex are shown in

tables 2 and 3. Analysis of Variance with repeated measures and returning to the values of F calculated in Huynh-Feldt Epsilon in accordance with the significant test of Mauchly's. Test Sphericity approved at the statistically significant level that with increasing the concentration of each of the treatments, the viability of MRSA was decreased (P<0.05).

Table 2.Comparison of statistical indicators of MIC of the study treatments against *MRSA* strains

strain of Methicillin Resistant Staphylococcus Aureus intervention	Statistic of MIC	standard strain	clinical strain	all strain	P Sign test
Satureja mutica fisch extract	Mean±SD	3000	1714±544.7	1800±621	0.0001 S
	Median	3000	1500	1500	
Zinc oxide nanoparticle	Mean±SD	40	24.29±8.52	25.33±9.15	0. 001 S
	Median	40	20	20	
Zinc complex	Mean±SD	20	27.14±9.95	26.67±9.76	0.025 S
	Median	20	20	20	
synergism of extract & nanoparticle	Mean ± SD of extract	750	428.6±136.18	450±155.26	0.001 S
	Median of extract	750	375	375	
	Mean ± SD of nanoparticle	10	6.07±2.13	6.33±2.29	0.001 S
	Median of nanoparticle	10	5	5	
synergism of extract & nanocomplex	Mean ± SD of extract	750	428.6±136.18	450±155.26	0.001 S
	Median of extract	750	375	375	
	Mean ± SD of nanocomplex	5	6.79±2.49	6.67±2.44	0.025 S
	Median of nanocomplex	5	5	5	
Kruskal–Wallis test result of clinical strain		Chi-Square=93.23	df= 6	p= 0.0001 S	
Kruskal–Wallis test result of all strain		Chi-Square=99.51	df= 6	p= 0.0001 S	

Table 3. Statistical comparison of the zone of inhibition (ZOI) of *MRSA* strains in the presence of study treatments

strain of Methicillin Resistant Staphylococcus Aureus		standard strain	clinical strain	all strain	P Sign test
intervention	Statistic of ZOI				
Satureja mutica fisch extract	Mean±SD	15	14.14±0.36	14.2±0.41	0.0001 S
	Median	15	14	14	
Zinc oxide nanoparticle	Mean±SD	7	6.04±0.57	6.1±0.6	0.001 S
	Median	7	6	6	
Zinc complex	Mean±SD	11	10.43±0.65	10.47±0.64	0.011 S
	Median	11	10.5	11	
synergism of extract & nanoparticle	Mean±SD	13	11.36±0.49	11.47±0.64	0.001 S
	Median	13	11	11	
synergism of extract & nanocomplex	Mean±SD	14	11.57±0.94	11.73±1.1	0.001 S
	Median	14	11	11	
Kruskal–Wallis test result of clinical strain		Chi-Square=76.22	df= 6	p= 0.0001 S	
Kruskal–Wallis test result of all strain		Chi-Square=79.58	df= 6	p= 0.0001 S	

3.5. RT-PCR results on the status of inhibition of coagulase gene expression *in the presence of different treatments of study*

Confirmation of coagulase gene presented in fig. 3. RT-PCR results in fig. 4 showed that *coa gene* expression had dropped after treatment with *Satureja mutica* extract (Line 1 prior to treatment and Line 2 after treatment), but Lines 3 and 4 indicate that *arcC* gene expression has not changed before and after the treatment. Moreover, based on the observed results in fig. 5, *coa gene* expression after treatment with nanocomplex has slightly decreased. Real time RT_PCR is needed to achieve a better result (Line 1 before treatment and Line 2 after treatment) and *arcC* gene expression before and after treatment had not changed (Lines 3 and 4). Besides, Fig. 6 also represented no changes in the expression of *coa* and *arc* genes before and after treatment with *zinc oxide nanoparticles.* Before performing RT-PCR, for better comparing the semi-quantitative results, the concentration of samples before and after treatment were homogenized.

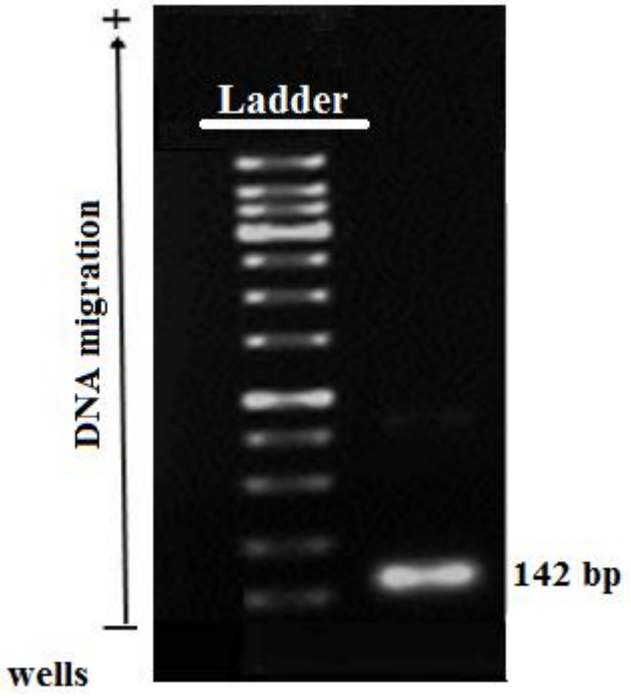

Fig 3.Confirmation of *coagulase* gene

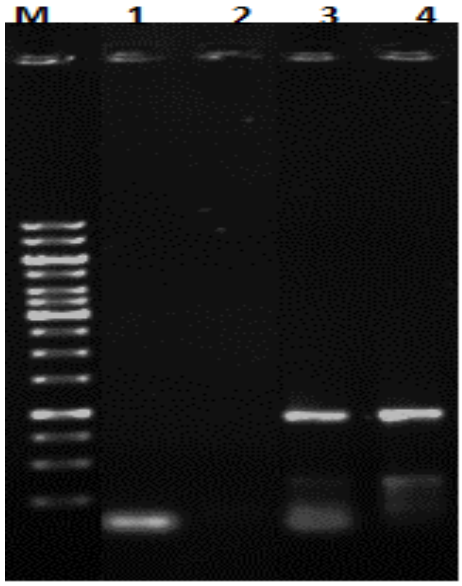

Fig 4. M: marker 1kbp , line 1: coa gene before treatment with satureja mutica ,line 2: coa gene after treatment with satureja mutica , line 3,4: arcC gene before and after treatment

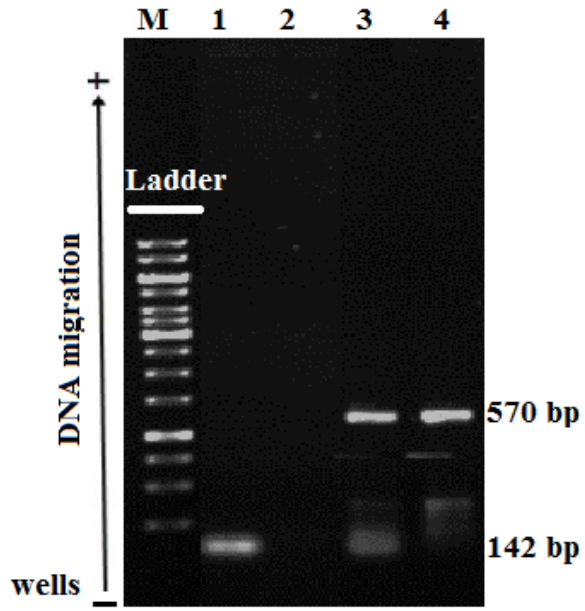

Fig 5. M: marker 1kbp , line 1: *coa* gene before treatment with zinc cimplex ,line 2: *coa* gene after treatment with zinc cimplex , line 3,4: arcC gene before and after treatment

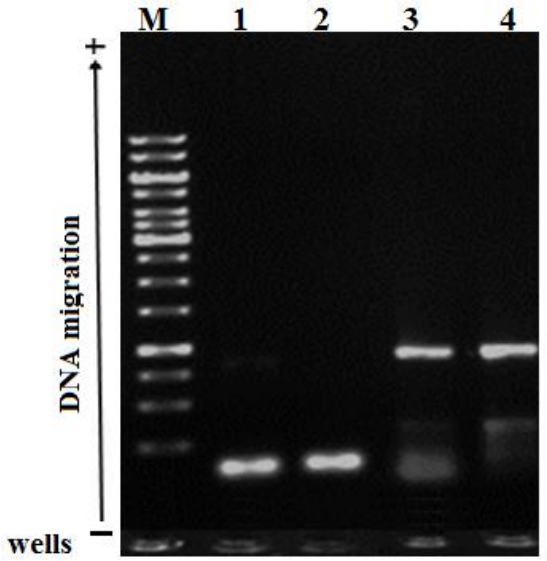

Fig6. M: marker 1kbp , line 1: *coa* gene before treatment with ZnO nano particle ,line 2: *coa* gene after treatment with ZnO nano particle , line 3,4: arcC gene before and after treatment

4. Discussion

S. aureus is one of the most important factors for nosocomial infections and community-acquired infections. Antibiotic resistance of S.aureus to Penicillin, and subsequently Methicillin underlies the resourcefulness to find other ways for treating infections caused by this pathogen 21. Among the antimicrobial agent's numerous researches were carried out in the field of medicinal plants and nanoparticles. In this study, the antimicrobial activity of plant medicine such as Satureja mutica and nanoparticle such as zinc oxide nanoparticle and zinc nano complex WERE tested against MRSA growth. The results of this study showed that Satureja mutica zinc nano complex and zinc oxide nanoparticles had inhibitory antibacterial property against MRSA. It also indicated that Satureja mutica has an inhibitory effect against pathogen coagulase gene expression but zinc nano complex and zinc oxide nanoparticles do not have any inhibitory effect against this gene. Several studies have been carried out on the antibacterial effects of medical plants.

S. aureus is one of the most important factors for nosocomial infections and community-acquired infections [26]. Antibiotic resistance of *S.aureus* to Penicillin, and subsequently Methicillin underlies the resourcefulness to find other ways for treating infections caused by this pathogen[21]. Among the antimicrobial agent's numerous researches in the field of medicinal plants and nanoparticles were carried out. In this study, the antimicrobial activity of plant medicine such as *Satureja mutica* and nanoparticle such as zinc oxide nanoparticle and zinc nanocomplex WERE tested against *MRSA* bacterium growth. The results of this study showed that *Satureja mutica* zinc nanocomplex and zinc oxide nanoparticles had inhibitory antibacterial property aginsr *MRSA* and also *Satureja mutica* has an inhibitory effect against pathogen *coagulase gene* expression but zinc nanocomplex and zinc oxide nanoparticles do not have any inhibitory effect against this gene. Several studies have been carried out on the antibacterial effects of medical plants. Among medical plants, only some studies have been done on essential oils of the Savory family but the antibacterial effect of zinc oxide nanoparticles, Satureja mutica extract and zinc complex was not found. In a study by Hadian et al. [19] antimicrobial effects of essential oils of Savory family strains were investigated. The results showed that the chemical compositions of the essential oil of these plants are similar to each other and all had antibacterial properties. Another study carried out by Jalalvandi et al. [20] the inhibitory effect of Satureja khuzestanica was investigated on the exoenzyme S, exotoxin A, secretory systems and efflux pumps in the antibiotic resistance of P. aeruginosa by

semi-quantitative RT-PCR technique, and it was found that this plant has the inhibitory effect against the mentioned genes.

Among these medical plants, only some work has been done on essential oils of the Savory family but the antibacterial effect of zinc oxide nanoparticles, *Satureja mutica* extract and zinc complex was not found. In a study by Hadian *et al*. [27] antimicrobial effects of essential oils of Savory family strains were investigated. The results showed that the chemical compositions of the essential oil of these plants are similar to each other and all had antibacterial properties. Another study carried out by Jalalvandi *et al*. [28] the inhibitory effect of Satureja khuzestanica was investigated on the exoenzyme S, exotoxin A, secretory systems and *efflux pumps* in the *antibiotic* resistance of *P. aeruginosa* by semi-quantitative RT-PCR technique, and it was found that this plant has the inhibitory effect against the mentioned genes. In Azam *et al.* [29]study, the antibacterial effect of a few nano-oxides was investigated on gram- positive and gram- negative bacteria. The results showed that zinc oxide nanoparticles had a better effect on both gram positive and gram negative bacteria. Raghupathi *et al.* [30] examined the growth inhibition and the mechanism of the effect of zinc nanoparticles on bacteria and indicated that the antimicrobial activity of zinc oxide nanoparticles due to the production of reactive oxygen and accumulation of nanoparticles in the cytoplasm or the outer membrane of bacteria. Due to the high resistance of *MRSA* compared to other drugs and the importance of this bacterium in *nosocomial infections* and burns, most pathogens are observed in immunocompromised patients, finding a safe and effective therapeutic supplement is necessary. Therefore, the identification of effective compounds that inhibit pathogenic genes of this bacterium is crucial. It is worth noting that determining the inhibition zone diameter is carried out through disk diffusion method, and considering that nanoparticles have little ability to release and with regard to the superiority of the MIC indicator in the determination of the antibacterial effects. It should be acknowledged that zinc nanocomplex compared to zinc oxide nanoparticle and *Satureja mutica* extract has less MIC in the level of significance and the simultaneous use of *Satureja mutica* extract with nanocomplex or zinc oxide nanoparticle in clinical strains of *MRSA* has created more powerful antibacterial effects than that of these simultaneous treatments at the standard strains ($P<0.05$). Accordingly, it must be noted that the simultaneous use of this extract along with zinc nanocomplex or zinc oxide nanoparticle increases the anti-bacterial effect and has stronger antibacterial effects than antibiotics to which these bacteria have become resistant and can be a good alternative to these antibiotics in the treatment of infections caused by *MRSA*. As the result showed, *Satureja mutica* extract has a significant inhibitory effect on the expression of *coagulase gene* in bacteria under study; thus, with studying this extract on other *virulent genes of MRSA and studying the obtained results, it can be use of this extract in the future with more studies as a treatment or therapeutic supplement.*

5. Acknowledgement

This Research was financially supported by deputy vice-chancellor for research affairs of Yasuj University of Medical Sciences (93.12.03.07). We thank the medical and nursing staff of Shahid Beheshti and Imam Sajjad hospitals of Yasuj for their help with sample collection

6. Conflicts of interest

The authors declare that there are no conflicts of interest

7. Ethical approval

This article does not contain any studies with animals performed by any of the authors.

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