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ORIGINAL ARTICLE

MIC and MBC of Honey and Gold Nanoparticles against methicillin-resistant (MRSA) and vancomycin-resistant (VRSA) coagulase-positive *S. aureus* isolated from contagious bovine clinical mastitis

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Abstract *Staphylococcus aureus* is one of the major causative agents of the bovine clinical mastitis. This study aimed to isolate and identify *S. aureus* from cases of bovine clinical mastitis followed by phenotypic detection of MRSA and VRSA. The genotypic detection of MRSA was done through PCR detection of the resistance *mecA* gene. Furthermore, this study aimed to investigate the in vitro MIC and MBC of the *Dodonaea angustifolia* plant extract, Honey, and AuNPs against the clinically isolated MRSA and VRSA. Of 93 mastitis milk samples examined, 54 (58.1%) *S. aureus* were isolated and identified {CP *S. aureus* = 46 (85.2%) and CN *S. aureus* = 8 (14.8%)}. The whole MRSA, VRSA, MSSA, and VSSA detected were 19 (35.2%), 7 (13%), 35 (65%), and 47 (87%) respectively. The mean counts of *S. aureus* were between $8.6 \times 10^4 \pm 3.5 \times 10^5$ CFU/ml. The oxacillin and vancomycin MICs against MRSA and VRSA respectively, were >256 μg/ml. AuNPs sized 30 nm produce observable in vitro anti-MRSA and anti-VRSA activities. Imtenan® citrus blossom honey has also antibacterial activities against MRSA and VRSA with general MBC and MIC range values were observed at a concentration of 0.625, 1.25, 2.5, and 5 (%v/v). In the present study, the most significant result obtained when AuNPs was mixed with Imtenan® citrus blossom honey (1:1 = v:v) with the best MBC was observed at the concentration of 0.56×10^9:0.3 (NP/ml: honey %v/v).

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1. Introduction

Mastitis is the most expensive problem facing the dairy industrial communities. Globally, the estimated economic losses due to mastitis reached about 533 billion $ [1]; the estimated economic loss of milk per cow per one lactation cycle due to mastitis is 70%, while it was 14% due to premature culling; on the other hand, it was 7% due to the exclusion of the mastitis milk and finally it was 8% due to the cost of the veterinary medication, of the total losses reported worldwide [2–4]. *Staphylococcus aureus* (*S. aureus*) is considered one of the main worldwide causative agents of 40–70% of contagious bovine mastitis [2,3]. Moreover, *S. aureus* is known worldwide as a toxigenic foodborne bacteria which is considered a dangerous threat to human life, because if *S. aureus* counts reached inside the food like milk to 10^5–10^6 CFU/ml or gm at temperatures between 10 °C and 46 °C, then it can be able to secrete dangerous heat-stable enterotoxins [5]. Furthermore, *S. aureus* has the ability to convert to a multi-drug resistant *S. aureus* knowing worldwide as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) [6,7]. MRSA is known worldwide as a multi-drug resistance acquired hospital pathogen, but recent reports revealed that MRSA was associated with cases of bovine mastitis [8]. Vancomycin was considered the drug of choice to overcome MRSA infection, but recently in 1997, MRSA that becomes intermediate susceptibility or resistant to vancomycin (VRSA) was begun to appear, meaning that MRSA can be a vancomycin resistance (MRSA + VRSA) [9]. The emergence of MRSA and VRSA in the cases of mastitis and its return harmful effect on the human being, with increasing failure in their treatment, and the associated high morbidity and mortality within both human and animals, all of that, raised a necessity to experimentally searching for a new therapeutic anti-MRSA and VRSA agents.

Honey consists mainly of sugars, water, minerals, and vitamins, especially B complex and vitamin C. Honey has been orally administrated for years due to their nutritional and therapeutic values; honey possesses anti-oxidant, anti-inflammatory, and anti-bacterial properties. Honey can overcome the multi-drug resistant bacteria [10]. Moreover, honey was able to overcome MRSA biofilms [11]. Experimentally, a significant decline in total bacterial counts in bovine subclinical mastitis was noticed after intra-mammary infusion by diluting honey [12]. Indeed, honey from all over the world have potent bactericidal activities and can reverse the antimicrobial resistance patterns [13].

Dodonaea angustifolia (*D. angustifolia*), which is a commonly used medicinal plant in clinically traditional medicine, has a wide range of therapeutic medical applications against various diseases, including malaria, viral, *Candida* [14–16], tuberculosis, pneumonia [17,18], and HIV diseases [19]. Furthermore, *D. angustifolia* demonstrated antimicrobial activity against Gram-negative (*E. coli*) and Gram-positive (*S. aureus* and *Bacillus pumilus*) bacteria as well as the fungus *Saccharomyces cerevisiae* [20].

Gold nanoparticles (AuNPs) have an antibacterial effect against most fungal and multi-drug resistant bacteria [21,22]. Indeed, AuNPs were able to overcome *S. aureus* [23]. The main advantage of AuNPs is that they have a low toxicity compared to various nanoparticles [24].

The aim of the present study is to isolate and identify *S. aureus* from cases of bovine contagious clinical mastitis followed by MRSA and VRSA detection. To the best of our knowledge, the present study is the first study that will reveal the in vitro MIC and MBC of *D. angustifolia* plant extract, Honey, and AuNPs against the clinically isolated MRSA and VRSA from contagious clinical mastitis milk samples.

2. Materials and methods

2.1. Sample collected

A total of 93 bovine contagious clinical mastitis milk samples were collected from different rural areas and veterinary units throughout Egypt. All samples were collected and examined during the period of December 2014 through September 2015. The samples were collected aseptically; the udder surfaces were washed thoroughly with distilled water, dried with cotton, and then the first two streams of milk were discarded and the third stream (≈10 mL) was collected in a sterile 50 ml falcon tube and transported in an insulated ice box within 4 h of the collection to the Microbiology and Immunology Department, Veterinary Division, National Research Centre, Cairo, Egypt, where it was analyzed immediately for the presence of *S. aureus*.

2.2. Sample preparation for the isolation and identification of S. aureus

The milk samples were incubated for 24 h at 37 °C and then centrifuged at 3000 rpm for 20 min, the supernatant was then discarded and the resulting pellet was taken and inoculated into 5 ml Brain-Heart Infusion broth (BHI, Oxoid) followed by incubation at 37 °C for 24 h. After that, a loopful from each broth was streaked into three agar media: Mannitol-Salt agar (MSA, Oxoid), Baird-Parker agar (BP, Oxoid) supplemented with Egg Yolk-Tellurite Emulsion (SR0054, Oxoid) and Blood agar (Oxoid) supplemented with 5% sheep red blood cells, and all plates were then incubated aerobically at 37 °C for 24 h. *S. aureus* identifications were carried out through microbiological procedures: characteristic yellowish colonies surrounded by yellowish zones on MSA (due to mannitol fermentation), characteristic black colonies surrounded by hallow clear zones on BP agar, hemolysis on Blood agar. All characteristic colonies were purified and streaked into BHI slants to complete the following microbiological identification procedures: Gram's staining (gram positive cocci in grapes like clusters), coagulase (either positive or negative), positive catalase, positive Voges Proskauer, positive DNase, and a positive presence of clumping factor in dry spot *Staphytect Plus*® (A latex slide agglutination test, Oxoid). Finally, *S. aureus* identifications were confirmed through API-*Staph* identification Kit (bio-Merieux) according to the manufacturer's instructions. All the identified *S. aureus* strains were streaked into BHI slants for the next steps and stored also at −80 °C in a BHI broth supplemented with 15% glycerol for long-term storage.

2.3. Total S. aureus counts in the milk samples

The enumerations of *S. aureus* in the milk samples were performed on both MSA and BP agar supplemented with Egg Yolk-Tellurite emulsion as follows: one ml of the milk

sample was tenfold serially diluted using 9 ml sterile saline, and 100 µl of each dilution was then inoculated in triplicate onto the surface of each MSA and BP agar plates and was evenly distributed by a sterile glass rod spreader. The plates were left for one minute at ambient temperature, and then were incubated aerobically at 37 °C for 24 h. All characteristic colonies of *S. aureus* on MSA and BP agar were microbiologically identified as mentioned above and then were counted as *S. aureus* and the mean *S. aureus* CFU was determined.

2.4. Detection of MRSA and VRSA isolates

The antimicrobial susceptibility of the isolated *S. aureus* toward cefoxitin and vancomycin was performed by the Kirby-Bauer disk diffusion method to detect MRSA and VRSA isolates respectively. From the previously isolated *S. aureus*, a loopful from each BHI culture slants was inoculated into Mueller-Hinton broth (MH broth, Oxoid) followed by incubation at 37 °C for 24 h. The concentrations of these suspensions were adjusted to be equal to the 0.5 McFarland standards by adding a sterile saline. Test and standard tubes were compared against a white background with a contrasting black line and complete adjustment of the suspension concentrations was done by spectrophotometer to reach to an optical density of 0.10 at 625 nm (1×10^8 CFU/ml). A swab spreading of each 0.5 McFarland *S. aureus*-MH broth concentration was done onto the surface of the Mueller–Hinton agar medium (MH agar, Oxoid) supplemented with 5% defibrinated sheep blood, and then cefoxitin (30 µg, Oxoid) and vancomycin (30 µg, Oxoid) sensitivity disks were impregnated onto the surfaces of the MH agar. Plates were then incubated at 37 °C for 24 h, the zones of inhibition were measured, and the susceptibilities to both antibiotics were determined according to interpretive criteria provided by the National Committee for Clinical Laboratory Standards [25].

2.5. MIC determination of the graded concentration oxacillin and vancomycin antibiotic strips against MRSA and VRSA isolates

From the previously identified MRSA and VRSA isolates, a swab spreading of 0.5 McFarland concentration of each MH broth inoculums was done onto the surface of the MH agar medium supplemented with 5% defibrinated sheep blood as mentioned before, and then Graded Concentration Oxacillin and Vancomycin antibiotic strips (MICE strips®; Oxoid) were aseptically placed into the surfaces of the plates and left for 15 min at room temperature. The plates were then inverted and placed in the incubator at 37 °C for 24 h. The MIC will be the lowest oxacillin and vancomycin concentrations that inhibit the visible growth of MRSA and VRSA respectively.

2.6. Molecular identification of MRSA using polymerase chain reaction

All isolates showing resistance to oxacillin were subjected to polymerase chain reaction for the detection of *mecA* gene specific for MRSA molecular detection as described previously by [26]; *mecA* gene was amplified using the following primer sequence: mecA-F:50-AAAATCGATGGTAAAGGTTGGC-30 and mecA-R:50-AGTTCTGCAGTACCGGATTTTGC-30. From the previously identified MRSA BHI slants, a loopful from each culture was inoculated into BHI broth followed by incubation at 37 °C for 24 h. The genomic DNA was extracted using DNA Purification Kit (Qiagen) according to the manufacturer's instructions. The purity of the extracted DNA was measured using a spectrophotometer (the OD_{260}/OD_{280} ratio of the extracted DNA was about 1.8). The extracted DNA was stored at −20 °C. In the PCR analysis, the amplification was performed in a total reaction volume of 25 µl containing 2 µl of 5.0 ng genomic DNA/µl, 1 µl of each primer pair of 150 Pico mol/reaction, 0.5 µl Taq DNA Polymerase of 5,000 µ/ml (Qiagen), 5 µl of PCR buffer (50 mM KCl, 10 mM tris-HCl (pH 8.3), 5.5 mM $MgCl_2$) and 1 µl of 10 mM deoxynucleotide triphosphate mixture of 200 µM each dATP, dCTP, dTTP and dGTP and finally adjusted to a final volume of 25 µl by nuclease free water in the PCR tube. After mixing, the mixtures were overlaid with 40 µl mineral oil to avoid evaporation; then, the tubes were placed in a programmable thermal cycler (PTC100 Mil Research, USA) and the amplification was performed under the cycling conditions: initial denaturation at 95 °C for 10 min, 35 cycles of amplification (denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min), and a final extension at 72 °C for 10 min. The PCR products were stored at −20 °C until use. The PCR products (533 bp) were separated by the electrophoresis at 100 volts for 45 min in a horizontal 1.5% agarose gel (Sigma–Aldrich) stained with 1% ethidium bromide (Sigma–Aldrich) using a running buffer consisting of Tris–acetate-EDTA (1X TAE) (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid; pH 8.0). The presence of specific amplified DNA bands was detected by visualization under UV light at a wavelength 421 nm in comparison with a molecular weight standard GeneRulerTM 100-bp DNA Ladder (Fermentus). MRSA ATCC 43300 was used as a positive control.

2.7. Bacterium inoculum preparations for the MIC and MBC experiments

From the previous identified MRSA and VRSA isolates, eight isolates (four isolates were MRSA + VRSA and another four isolates were MRSA + VSSA) were chosen for the in vitro MIC and MBC experiments in the present study. With a sterile loop, a loopful from the eight chosen BHI slants was inoculated into the MH broth and then incubated with shaking at 37 °C overnight for 24 h. The concentrations of each suspension were adjusted to be equal to the 0.5 McFarland standards as mentioned before. Finally, the culture suspensions were then diluted to be equal to the concentration of 5×10^5 CFU/ml.

2.8. Antibacterial preparations for the MIC and MBC experiments

2.8.1. Dodonaea angustifolia

The plant extraction was carried out according to the method described in [27] with some modification; briefly, the plant was collected, washed with tap water, again rinsed with distilled water, dried in drying hot air oven at 40 °C, and grinded. 50 grams of the shade resulting material was added to 500 ml absolute ethanol for 24 h with continuous shaking. The extract was then filtered through a 22 µm filter paper, and then the resulting filtrate was dried using a rotatory evaporator at

40 °C. The previous procedure was repeated three times, and then 100 mg of the resulting powder material was dissolved in 1 ml dimethyl sulfoxide solvent (DMSO:Water, 2:4 v/v) to prepare the stock solution of 100 mg/ml. Then this stock will be 10-fold diluted to obtain 10 mg/ml and then twofold serial dilutions were done using MH broth to prepare the following concentrations (5, 2.5, 1.25, 0.625, 0.3, 0.15, 0.08, 0.04, 0.02 and 0.01 mg/ml).

2.8.2. Honey

One 500 ml bottle of processed well-identified honey labeled as "100% natural citrus blossom honey" which is authorized by the Egyptian Ministry of Health, was purchased from Imtenan® health stores in Nasr City, and used in the MIC and MBC experiment. 10-fold dilution was done first to 1 ml of honey (100% v/v) to obtain 10%/ml v/v and then twofold serial dilutions were done using MH broth to prepare the following concentrations: 5, 2.5, 1.25, 0.625, 0.3, 0.15, 0.08, 0.04, 0.02 and 0.01%/ml v/v.

2.8.3. Gold Nanoparticles

Stabilized suspension of AuNPs of an analytical grade in citrate buffer (size of 30 nm) (concentration of 1.79×10^{11} nanoparticles/ml) was purchased from Sigma-Aldrich. 10-fold dilution was done first to 1 ml of the stock nanoparticle solution to obtain 1.79×10^{10} nanoparticles/ml and then twofold serial dilutions were done using MH broth to prepare the following concentrations: 9×10^9, 4.5×10^9, 2.24×10^9, 1.12×10^9, 5.6×10^8, 2.8×10^8, 1.4×10^8, 7×10^7, 3.5×10^7 and 1.75×10^7 nanoparticles/ml.

2.9. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of *D. angustifolia*, Gold Nanoparticles, Honey against clinically isolated MRSA and VRSA isolates

The antimicrobial effectiveness of *D. angustifolia* extract, honey, and AuNPs against the eight chosen isolates (four isolates MRSA + VRSA and another four isolates MRSA + VSSA) were analyzed through determination of the minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and MBC/MIC ratio values through application of the broth micro-dilution method using 96-well micro-titer plates as follows, to each 50 µl of the antibacterial agent dilution vertically, 50 µl of the adjusted bacterial concentration inoculums (5×10^5 CFU/mL) was added horizontally in the 96-well micro-titer plates, and the growth control wells contained MH broth medium with tested bacterial concentrations in order to check the bacterial viability while the sterility control wells contained only a sterile MH broth in order to check the sterility of the medium used. The plates were then covered to ensure that the bacteria were not dehydrated. The plates were then incubated at 37 °C for 18–20 h. The lowest concentration of each antibacterial agent that inhibited the bacterial growth was then considered as the MIC [28]. After the MIC determination, aliquots of 100 µL from each well that does not show any bacterial growth after incubation were streaked onto BHI agar plates followed by incubation at 37 °C for 20 h. The lowest concentration that kills 100% of the initial bacterial population showing no colonies on the BHI agar was recorded as the MBC.

2.10. Statistical analysis

Quantitative data of the MIC and MBC in each antimicrobial agent were statistically represented in terms of minimum, maximum, median, and range (95% CI). Comparison between the two nonparametric groups (gold nanoparticles and honey both in alone form and in mixed form) in the present study was done using Mann-Whitney Test. A probability value (p value) less than or equal to (0.05) was considered significant. All statistical analyses were performed using statistical software SPSS (Statistical Package for Social Science) statistical program version (16.0). Graphs were done using Microsoft Excel program version 2010.

3. Results

3.1. *S. aureus* isolation

Out of the 93 contagious clinical mastitis milk samples examined, 54 (58.1%) *S. aureus* were identified as the causative agent of mastitis. The number of the coagulase positive (CP) and coagulase negative (CN) *S. aureus* was 46 (85.2%) and 8 (14.8%) respectively.

3.2. Total *S. aureus* counts in the milk samples

The mean counts of *S. aureus* recovered from the 54 *S. aureus* positive mastitic milk samples examined were between $8.6 \times 10^4 \pm 3.5 \times 10^5$ CFU/ml.

3.3. Detection of MRSA and VRSA isolates by the Kirby-Bauer disk diffusion method

The eight CN *S. aureus* isolates were neither resistant to cefoxitin nor vancomycin. Out of the 46 isolated CP *S. aureus*, 7 (13%) isolates were resistant to both cefoxitin and vancomycin (MRSA + VRSA) while 12 (22.2%) isolates were cefoxitin resistant but vancomycin sensitive (MRSA + VSSA). On the other hand, the remaining 27 CP *S. aureus* were sensitive to both cefoxitin and vancomycin (MSSA + VSSA) with an incidence of 50%. The whole MRSA, VRSA, MSSA, and VSSA detected according to the whole 54 *S. aureus* detected were 19 (35.2%), 7 (13%), 35 (65%), and 47 (87%) respectively.

In accordance with the whole mastitis milk samples examined (no = 93), the percentages of MRSA, VRSA, MSSA, and VSSA were 20.4, 7.5, 37.6, and 50.5% respectively while the percentage of both (MRSA + VRSA) and (MRSA + VSSA) were representing 7.5 and 12.9% respectively.

3.4. The MIC of the graded concentration oxacillin and vancomycin antibiotic strips against MRSA and VRSA isolates

All the previous identified MRSA and VRSA isolates by the disk diffusion method were subjected to the graded concentration oxacillin and vancomycin antibiotic strip examination, respectively, and the obtained MICs were >256 µg/ml for both oxacillin and vancomycin against MRSA and VRSA respectively.

3.5. Molecular identification of MRSA using polymerase chain reaction

PCR amplification of the specific *mecA* gene revealed that, all the previous identified MRSA isolates were positive for the presence of *mecA* gene as presented in Fig. 1.

3.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of the D. angustifolia, gold nanoparticles, honey against MRSA and VRSA isolates

Fig. 2(A) shows that *D. angustifolia* plant extract didn't produce any anti MRSA or VRSA effect since the bacteria remain viable for up to 48 h. However, Fig. 2(B–D) shows that the gold nanoparticles and Imtenan® citrus blossom honey both in a alone form and in mixed form have anti MRSA and VRSA effect and the MIC, MBC values, and MBC/MIC ratios of the them are presented in Table 1.

3.7. Statistical analysis

The statistical analysis of the MIC and MBC of each antimicrobial agent against two groups of bacteria (MRSA + VSSA and MRSA + VRSA) was represented in terms of minimum, maximum, median, range (95% CI), and P value as represented in Tables 2 and 3 and Diagrams 1-4. Tables 2 and 3 describe also the Mann–Whitney test (nonparametric test) between each antimicrobial agent in alone form and in a mixed form.

The statistical analysis showed significant difference between the effect of the Imtenan® Citrus Blossom honey in alone form and in the mixture form on the growth inhibition of MRSA + VSSA with the MIC P value was 0.017 (<0.05) and the MBC P value was 0.013 (<0.05), while in the case of MRSA + VRSA, the MIC P value was 0.025 (<0.05) and the MBC P value was 0.022 (<0.05) as represented in Table 2 and diagram Diagram 1 and 2.

Furthermore, the statistical analysis also showed significant difference between the effect of gold nanoparticles in alone form and in mixture form on the growth inhibition of MRSA + VSSA with the MIC P value was 0.015 (<0.05) and the MBC P value was 0.008 (<0.05) while in case of MRSA + VRSA, the MIC P value was 0.046 (<0.05) and the MBC P value was 0.040 (<0.05) as represented in Table 3 and Diagram 3 and 4.

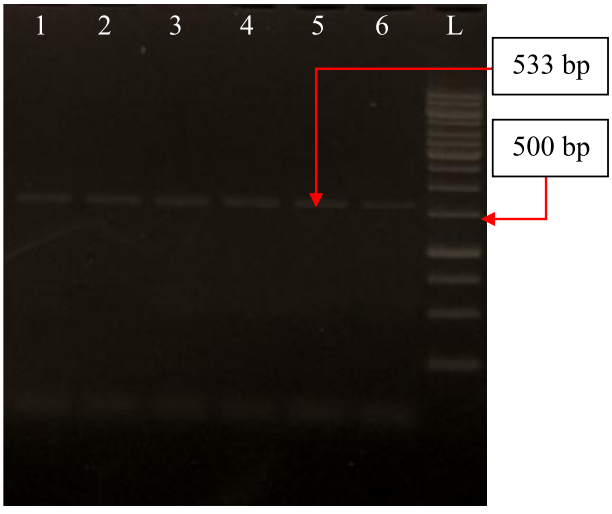

Figure 1 Amplification of 533 bp of *mecA* gene specific for MRSA molecular identification. Lanes 1–5: MRSA identified isolates, lane 6: MRSA ATCC 43300 as a positive control, lane L: a molecular weight standard GeneRulerTM 100-bp DNA Ladder (Fermentus).

4. Discussion

The early isolation and identification of the mastitis causative agent are very important in their prevention, treatment, and control [29]. The present study revealed a high percentage of mastitis caused by *S. aureus;* out of the 93 bovine contagious clinical mastitis milk samples examined, 54 (58.1%) *S. aureus* isolates were isolated and identified. Furthermore, there was a high percentage of coagulase positive *S. aureus* [CP] = 46 (85.2%) than coagulase negative [CN] *S. aureus* = 8 (14.8%). A previous study reported that, out of 420 mastitis milk samples examined, 84 (20%) *S. aureus* were detected; 50 (59.5%) and 34 (40.5%) were identified as coagulase-positive *S. aureus* and coagulase-negative *S. aureus* respectively [30]. Furthermore, 10.2% *S. aureus* were isolated from cases of bovine mastitis [31] which is less than our results. However, *S. aureus* were the causative agent of 11 (73.3%) clinical mastitis cows examined, and all of these isolates were identified as coagulase positive *S. aureus;* these results were higher than our study [32]. Moreover, 448 (50.1%) *S. aureus* were isolated from mastitic milk cases examined [29] which is nearly similar to the present result. Furthermore, the incidence of *S. aureus* isolated from mastitic dairy cows in another study was 52.5% [33] which is nearly similar to the present result another study.

Our study revealed also high counts of *S. aureus*; the mean counts were between $8.6 \times 10^4 \pm 3.5 \times 10^5$ CFU/ml which exceeded the limit set by the European Union Council Directive for direct human consumption in which the count should be less than $< 5 \times 10^2$ CFU/ml [34]. In agreement with the present study, a previous study reported that out of 35 milk samples examined, nearly 33 (94%) were contaminated with *S. aureus* with an average count 5.5×10^5 CFU/ml [35]. Another study also revealed that *S. aureus* counts in 930 milk samples examined were about 4.08 log CFU/ml [36]. Moreover, *S. aureus* counts in milk samples were in the range of 2.73–3.55 log CFU/ml [37]. In the present study, the presence of the performed *S. aureus* enterotoxin did not be investigated because the counts of *S. aureus* were sufficient enough for toxin production ($> 10^5$ CFU/ml) as previously mentioned by [5].

The high prevalence and count of *S. aureus* have recovered from the examined mastitic milk sample in the present study could be due to the poor hygienic practices which could transfer the infection from the infected mastitic animal to the healthy one; this could be occurred when both healthy and infected cows were milked together at the same place, time, milking equipment and devices, or by the same workers or owners. Furthermore, it could be contributed to inadequate personal hygiene. Several research articles indeed reported that the contagious mastitis could be a human introduced infection;

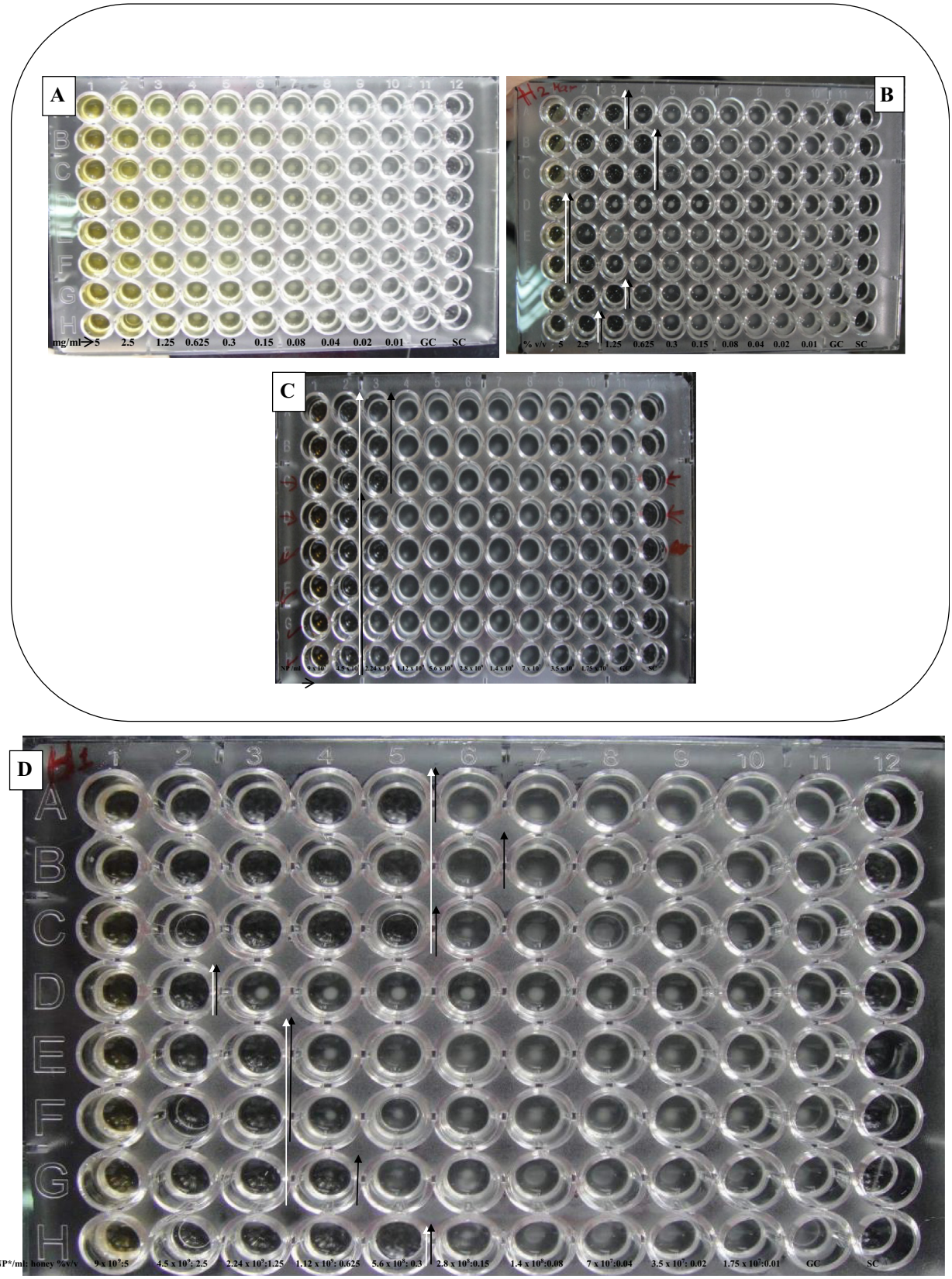

Figure 2 (A–D) Microtiter plates showing the MICs and MBC of (A): *D. angustifolia* plant extract, (B): Imtenan® citrus blossom honey, (C): gold Nanoparticles, (D): gold nanoparticles + Imtenan® citrus blossom honey (1:1 = v:v). (Rows A–G): MRSA, VRSA and VSSA as represented in Table 1, (columns 1–10): dilutions of each examined Anti MRSA and VRSA agents, (columns 11): GC (growth control wells), (columns 12): SC (sterility control wells) and the black and white arrows indicate the MICs and MBCs respectively against each isolate.

Table 1 MIC, MBC and MBC/MIC ratio determination of anti MRSA, VRSA and VSSA agents examined.

Rows	Bacterial isolates	Anti MRSA and VRSA agents examined											
		Gold nanoparticles (alone)			Imtenan® citrus blossom honey (alone)			Gold nanoparticles + Imtenan® citrus blossom honey (1:1 = v:v) (mixed together)					
								Gold nanoparticles (in mixture form)			Imtenan® citrus blossom honey (in mixture form)		
		MIC (NP/ml)	MBC (NP/ml)	MBC/MIC ratio	MIC (%v/v)	MBC (%v/v)	MBC/MIC ratio	MIC (NP/ml)	MBC (NP/ml)	MBC/MIC ratio	MIC (%v/v)	MBC (%v/v)	MBC/MIC ratio
A	MRSA + VSSA	2.24×10^9	4.5×10^9	2	1.25	1.25	1	0.56×10^9	0.56×10^9	1	0.3	0.3	1
B	MRSA + VSSA	2.24×10^9	4.5×10^9	2	0.625	0.625	1	0.28×10^9	0.56×10^9	2	0.15	0.3	2
C	MRSA + VSSA	2.24×10^9	4.5×10^9	2	0.625	0.625	1	0.56×10^9	0.56×10^9	1	0.3	0.3	1
D	MRSA + VRSA	4.5×10^9	4.5×10^9	1	5	5	1	4.5×10^9	4.5×10^9	1	2.5	2.5	1
E	MRSA + VRSA	4.5×10^9	4.5×10^9	1	5	5	1	2.24×10^9	2.24×10^9	1	1.25	1.25	1
F	MRSA + VRSA	4.5×10^9	4.5×10^9	1	5	5	1	2.24×10^9	2.24×10^9	1	1.25	1.25	1
G	MRSA + VRSA	4.5×10^9	4.5×10^9	1	1.25	1.25	1	1.12 x10^9	2.24×10^9	2	0.625	1.25	2
H	MRSA + VSSA	4.5×10^9	4.5×10^9	1	2.5	2.5	1	0.56×10^9	0.56×10^9	1	0.3	0.3	1

Table 2 The Mann-Whitney (Nonparametric Test) statistically analysis compared between the Imtenan® Citrus Blossom Honey (alone form) and Imtenan® Citrus Blossom Honey (mixture form) against two groups of bacteria examined (MRSA + VSSA and MRSA + VRSA) in terms of the minimum, maximum, median, and the P value of each parameter of the MIC, MBC and MBC/MIC.

Bacterial isolates	Parameters	Type	N	Min.	Max.	Median	Range (95% CI)	P value
MRSA + VSSA	MIC (%v/v)	Imtenan® Citrus Blossom Honey (Alone)	4	0.63	1.25	0.94	0.62 (−0.157–2.657)	0.017
		Imtenan® Citrus Blossom Honey (mixture form)	4	0.15	0.30	0.30	0.15 (0.143–0.382)	
	MBC (%v/v)	Imtenan® Citrus Blossom Honey (Alone)	4	0.63	1.25	0.94	0.62 (−0.157–2.657)	0.013
		Imtenan® Citrus Blossom Honey (mixture form)	4	0.30	0.30	0.30	0.00 (0.300–0.300)	
	MBC/MIC ratio	Imtenan® Citrus Blossom Honey (Alone)	4	1.00	1.00	1.00	0.00 (1.000–1.000)	0.317
		Imtenan® Citrus Blossom Honey (mixture form)	4	1.00	2.00	1.00	1.00 (0.454–2.046)	
MRSA + VRSA	MIC (%v/v)	Imtenan® Citrus Blossom Honey (Alone)	4	2.50	5.00	5.00	2.50 (1.079–7.046)	0.025
		Imtenan® Citrus Blossom Honey (mixture form)	4	0.63	2.50	1.25	1.87 (0.155–2.658)	
	MBC (%v/v)	Imtenan® Citrus Blossom Honey (Alone)	4	2.50	5.00	5.00	2.50 (1.079–7.046)	0.022
		Imtenan® Citrus Blossom Honey (in mixture form)	4	1.25	2.50	1.25	1.25 (0.568–2.557)	
	MBC/MIC ratio	Imtenan® Citrus Blossom Honey (Alone)	4	1.00	1.00	1.00	0.00 (1.000–1.000)	0.317
		Imtenan® Citrus Blossom Honey (mixture form)	4	1.00	2.00	1.00	1.00 (0.454–2.046)	

for example, a previous study reported that the workers or owners' hand has the largest impact on the high prevalence of mastitis especially those where the causative agent was MRSA [3].

CLSI guideline suggests the use of cefoxitin or oxacillin disk diffusion or minimum inhibitory concentration (MIC) as an alternative method for MRSA detection [34]. Molecular detection of the *mecA* gene and oxacillin resistance has been adopted also as a mean for MRSA detection [38]. MRSA detection cannot be based on either separate phenotypic or genotypic methods, but it must include both methods together [39]. So in the present study, cefoxitin and vancomycin Kirby-Bauer disk diffusion method followed by MIC determination of the oxacillin and vancomycin graded concentration strips

Table 3 The Mann-Whitney (Nonparametric Test) statistically analysis compared between the Gold Nanoparticles (alone form) and the Gold Nanoparticles (mixture form) against two groups of bacteria examined (MRSA + VSSA and MRSA + VRSA) in terms of the minimum, maximum, median, and the P value of each parameter of the MIC, MBC and MBC/MIC.

Bacterial isolates	Parameters	Type	N	Min.	Max.	Median	Range (95% CI)	P value
MRSA + VSSA	MIC (NP/ml)	Gold Nanoparticles (Alone)	4	2.24E + 09	4.50E + 09	2.24E + 09	2.26E + 09 (1.01E + 09–4.60E + 09)	0.015
		Gold Nanoparticles (in mixture form)	4	2.80E + 08	5.60E + 08	5.60E + 08	2.8E + 08 (2.67E + 08–7.13E + 08)	
	MBC (NP/ml)	Gold Nanoparticles (Alone)	4	4.50E + 09	4.50E + 09	4.50E + 09	0.00 (4.50E + 09–4.50E + 09)	0.008
		Gold Nanoparticles (in mixture form)	4	5.60E + 08	5.60E + 08	5.60E + 08	0.00 (5.60E + 08–5.60E + 08)	
	MBC/MIC ratio	Gold Nanoparticles (Alone)	4	1.00	2.00	2.00	1.00 (0.954–2.546)	0.186
		Gold Nanoparticles (in mixture form)	4	1.00	2.00	1.00	1.00 (0.454–2.046)	
MRSA + VRSA	MIC (NP/ml)	Gold Nanoparticles (Alone)	4	4.50E + 09	4.50E + 09	4.50E + 09	0.00 (4.50E + 09–4.50E + 09)	0.046
		Gold Nanoparticles (in mixture form)	4	1.12E + 09	4.50E + 09	2.24E + 09	3.38E + 09 (2.68E + 08–4.78E + 09)	
	MBC (NP/ml)	Gold Nanoparticles (Alone)	4	4.50E + 09	4.50E + 09	4.50E + 09	0.00 (4.50E + 09–4.50E + 09)	0.040
		Gold Nanoparticles (in mixture form)	4	2.24E + 09	4.50E + 09	2.24E + 09	2.26E + 09 (1.01E + 09–4.60E + 09)	
	MBC/MIC ratio	Gold Nanoparticles (Alone)	4	1.00	1.00	1.00	0.00 (1.000–1.000)	0.317
		Gold Nanoparticles (in mixture form)	4	1.00	2.00	1.00	1.00 (0.454–2.046)	

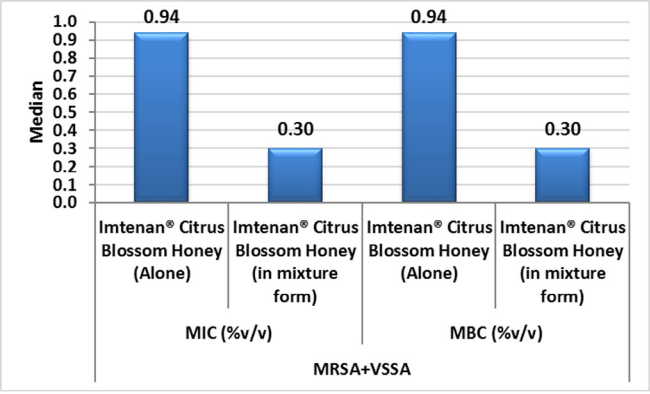

Diagram 1 The statistical analysis in terms of the median values of the MIC and MBC in the Imtenan® Citrus Blossom honey in alone form and in mixed form against MRSA + VSSA.

were used for MRSA and VRSA phenotypic detection respectively while MRSA genotypic detection was achieved by PCR amplification of the *mecA* gene.

In the present investigation, the eight isolated CN *S. aureus* were neither resistant to cefoxitin nor vancomycin. However, 121 coagulase negative [CN] *staphylococci* that were isolated from cases of bovine mastitis: of these isolates, 25 were classified as phenotypic MRSA while the other 96 isolates were classified as MSSA depending on the oxacillin susceptibility in the disk diffusion test [40].

In the present study, out of 46 isolated CP *S. aureus,* 35.2% and 13% were phenotypically identified as MRSA and VRSA respectively, and the percentage of both (MRSA + VRSA) and both (MRSA + VSSA) were 13 and 22.2% respectively. While MSSA and VSSA percentage was relatively high as it represents 65 and 87% respectively depending on the Kirby-Bauer disk diffusion and the MICs for both oxacillin and vancomycin graded concentration strips against MRSA and VRSA respectively that were >256 μg/ml. Moreover, the genotypic detection of MRSA indicates a complete correlation between phenotypic and genotypic resistance patterns; as all of the phenotypic identified MRSA isolates 19 (35.2%) were positive for the presence of *mecA* gene (100%).

Many research articles investigated the presence of MRSA, VRSA, MSSA, and VSSA within mastitic milk samples examined; for example, out of 53 *S. aureus* detected in mastitic milk, 25 (47.2%) were cefoxitin resistant indicating MRSA percentage [32] which is slightly higher than our result

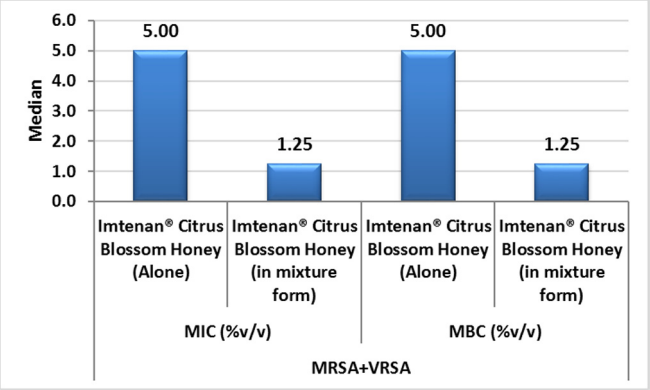

Diagram 2 The statistical analysis in terms of the median values of the MIC and MBC in the Imtenan® Citrus Blossom Honey in alone form and in mixed form against MRSA + VRSA.

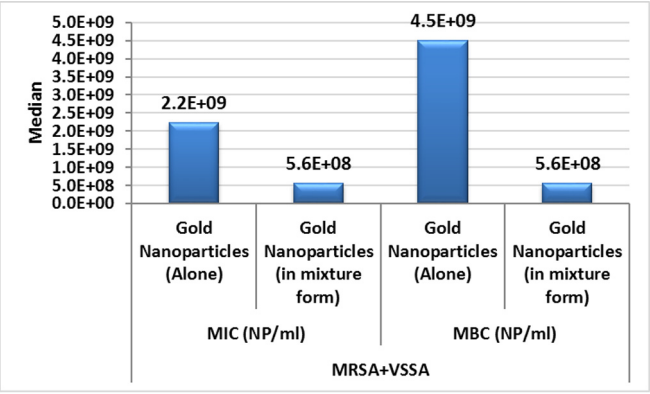

Diagram 3 . The statistical analysis in terms of the median values of the MIC and MBC in the gold nanoparticles in alone form and in mixed form against MRSA + VSSA.

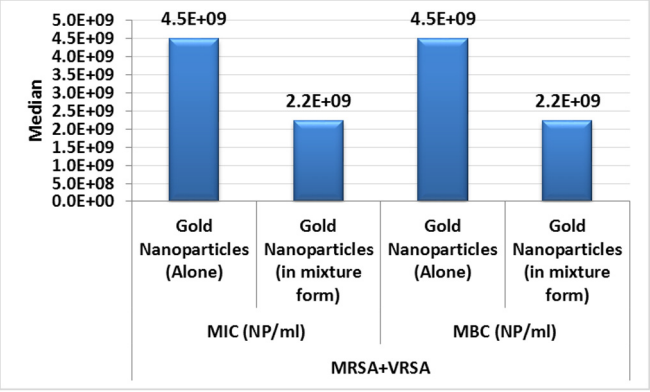

Diagram 4 The statistical analysis in terms of the median values of the MIC and MBC in the gold nanoparticles in alone form and in mixed form against MRSA + VRSA.

(MRSA = 35.2%). Also, 100% of the isolated methicillin-resistant *S. aureus* of the examined milk samples were resistant to oxacillin (1 μg) while no one was resistant to vancomycin (30 μg) by the disk diffusion method indicating MRSA + VSSA isolation [41]. Furthermore, oxacillin graded antibiotic strips antibiotics were also used by Kamal and his coauthors [34] against MRSA strains, in which all of the MRSA isolates showed resistance against oxacillin (100%) and the MIC was also >256 μg/ml which is similar to the present result. Furthermore, 41 (42.3%) and 56 (57.7%) *S. aureus* isolates were identified as MRSA and MSSA respectively (of the total isolated *S. aureus*) that were isolated from mastitis cases based on oxacillin susceptibility testing and PCR molecular identification of the *mecA* that allows the discrimination between MSSA and MRSA [31]. Moreover similar to our result, a previous study in Algeria reported that, 21 *Staphylo-*

cocci (10 *Staphylococcus aureus* and 11 coagulase negative *Staphylococci*) were isolated from bovine mastitis milk and these isolates were further in vitro phenotypic and genotypic resistance investigated; regarding the agar diffusion test, a total of 17 (80.95%) were identified as MRSA based on resistance to oxacillin and cefoxitin and all of them contain the *mecA* gene by PCR analysis similar to the present results while 76.19% were resistance to vancomycin [42] indicating (MRSA + VRSA) percentage which is more higher (76.19%) than ours (13%). On the other hand, MRSA and MSSA percentages from bovine mastitis were 2.27 and 95.45 respectively and none of the MRSA isolates were found to be resistant to vancomycin indicating (MRSA + VSSA), although it possessed both methicillin-resistant gene (*mec-A*) and vancomycin-resistant gene (*van-A*) [43] which is similar to our results. On the other hand, 103 *S. aureus* isolates were obtained from bovine mastitis in China; 49 (47.6%) were found to be *mecA*-positive, indicating the high incidence of MRSA. However, 37 of the 49 *mecA*-positive isolates were susceptible to oxacillin as determined by antimicrobial susceptibility assays [44] which is on contrary to our study.

The high prevalence of MRSA and VRSA noticed in the present study could be attributed to that, VRSA can be generated through horizontal transfer of a plasmid-born *van*-gene transposon from vancomycin-resistant *Enterococcus* to *S. aureus* across the genus barrier [7]. On the other hand, MRSA has the ability to synthesize a penicillin binding protein (PBP2a) which is encoded by the *mecA* gene. This *mecA* gene is responsible for overcoming the inhibitory effect of β-lactam antibiotics through prevention of their attachment to MRSA cell wall proteins [32,7,35]. Furthermore, MRSA has the ability for the beta-lactamase enzyme production, and this enzyme in turn has the ability to inactivate the Beta-lactam antibiotics such as penicillin and closely related antibiotics including, methicillin, oxacillin, cefoxitin, cloxacillin, dicloxacillin, flucloxacillin, nafcillin, and temocillin. Indeed, 50% of the *S. aureus* caused mastitis is occurred by beta-lactamase producing *S. aureus* [45]. Moreover, MRSA was found to be associated with previous treatment history of the animal with cefoxitin resistance [32].

Regarding our knowledge, the present study is the first study revealing the MIC and MBC of *D. angustifolia* plant extract, honey, and AuNPs against MRSA and VRSA isolates clinically isolated from contagious clinical mastitis milk samples investigated. In the present study, *D. angustifolia* plant extract did not produce any observable anti MRSA or VRSA effect since the bacteria remained viable even after 48 h as shown in Fig. 2(A). We did not find research articles demonstrated the effect of *D. angustifolia* on MRSA and VRSA except one previous study investigated the effect of *D. angustifolia* on *S. aureus* which reported that, the exudates of *D. angustifolia* demonstrated antimicrobial activities against *S. aureus* [20]. Despite that, when the authors made a chromatographic separation of the *D. angustifolia* exudates, some yielding compounds were inactive against *S. aureus* even at the highest concentration tested [20,46]. Moreover, a previous study demonstrated that *D. angustifolia* can display its antimicrobial effectiveness through penetration of the bacterial cell membranes [47] so our negative result may be due to that the MRSA or VRSA has the ability to inhibit the *D. angustifolia* antimicrobial agent penetration to the bacterial cell wall proteins.

Our study demonstrates also an observable anti-MRSA and VRSA effect of the 30 nm sized AuNPs with the main 100% MBC observed at a concentration of 4.5×10^9 (NP/ml) and also the main MIC was observed at a concentration of 4.5×10^9 (NP/ml) followed by 2.24×10^9 (NP/ml) and the MBC/MIC ratio was mainly 1 followed by 2 as presented in Table 1 and Fig. 2C. Indeed, the antibacterial effect of AuNPs against *S. aureus* increased with the increased dose of AuNPs; however, the small size 6–34 nm AuNPs showed 22.4% reduction in the counts of *S. aureus* [48]. Also, another study documented that, the 10 nM AuNPs were active against *S. aureus* with 5–10 μg/ml MIC range value, while it was 2.5–10 μg/ml in case of 20 nM AuNPs [49]. Moreover, Li and his coauthors reported that AuNPs were effective in treating MRSA, with MICs of 32 nM [50]. Furthermore, the antimicrobial activity of AuNPs against MRSA was documented in the form of measured inhibition zones which was between 16 and 17 mm [51].

The observed anti-MRSA and VRSA effect of the gold nanoparticles in this study could be due to the damaging or rupture of the bacterial cell outer membrane. Indeed a previous study noted that the AuNPs can interact with the cell membrane of gram-positive bacterium resulting in the formation of distinct aggregation patterns and lysis of the bacterial cell [52]. Furthermore, the blubbing caused by cationic AuNPs induced bacterial membrane damage [53]. Indeed, the AuNPs particles interact with the building elements of the bacterial cell outer membrane, causing structural changes, degradation and finally cell death [54]. Moreover, the small size of the AuNPs facilitated its entry to the bacteria cell. Indeed, the AuNPs possess a smaller size 250 time than the bacterial cell as it may reach to 15 nm [54]. Furthermore, the AuNPs possess a well-developed surface chemistry and chemical stability facilitating it to maintain a constant shape and size in any solution, making them easier to interact with the bacterial cell [54]. Moreover, the antimicrobial activity of AuNPs may be due to their larger total surface area per unit volume [55,56]. Over that, the AuNPs have the ability to alter the bacterial cell metabolisms; as AuNPs can modify the membrane potential and inhibit the adenosine triphosphate (ATP) synthesis resulting in general decline in the bacterial cell metabolism. Moreover, AuNPs also can inhibit the ribosomal subunit concerning tRNA binding resulting in a collapse of biological process [57].

From the results presented in Table 1 and Fig. 2 in the present study, it is clear that the Imtenan® citrus blossom honey has antibacterial activities against MRSA and VRSA with general MBC and MIC range values were observed at concentrations of 0.625, 1.25, 2.5, and 5 (%v/v) while the general MBC/MIC ratio was 1. Many previous research articles studied the effectiveness of honey against MRSA [58,59,11,60,61]. For example, a previous study revealed that, the MIC of honey was between 15 and 20% v/v for the MRSA isolates [10]. Furthermore, five types of honey against MRSA were found to be active against MRSA but with varying degrees with general MIC and MBC range values observed between 8.33 and 33.3% (w/v) [58]. A very common therapeutic honey known worldwide was Manuka honey, which was found to have ability in complete elimination of 70% of MRSA [62]. Also, a complete elimination of MRSA was achieved after Manuka honey topical application [63]. The anti-MRSA and VRSA effectiveness of honey are attributed to several mechanisms; for example, honey can reverse the antimicrobial resistance

feature of MRSA and VRSA [13,64] through downregulation mechanism of *mecR1* gene product, a transducer which contributed to antibiotic resistance in MRSA [13]. Also, honey has the ability to produce bactericidal compounds such as hydrogen peroxide, polyphenols, phenolic acids, and flavonoids [58,65,66,59,67]. Indeed, honey produces hydroxyl radicals, which degrade the bacterial cell DNA, resulting in bacterial cell destruction in a dose dependent manner [59,8]. Furthermore, the high osmolarity of honey (due to high sugar content) has the ability to reduce the water activity in the bacterial cell resulting in bacterial growth inhibition [58,67]. Moreover, the low pH of honey facilitating its destruction features against MRSA and VRSA [67]. Finally, the antibacterial effects of honey may be due to honey lysosomal contents [68] or may be due to cytokine production [69]. Allah has also told us in Holy Qur'an:

"وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا ۚ يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِّلنَّاسِ ۗ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ"

Which means that Allah inspired to the bee to make their home in the mountains, in the trees, and in the places done by the men and then Allah ordered the bees to eat from all the fruits following the ways of Allah. Then Allah made a variety of colored drinks (honey) which have an easy emerges from bee's abdomen wherein is a healing for mankind, which is a sign for those who reflect (An-Nahl: 68&69, Holy Qur'an).

Furthermore, the statistically analysis showed significant difference between the Imtenan® Citrus Blossom honey and the AuNPs both in alone form and in mixture form with general P value < 0.05 in either MIC or MBC. It is obvious from Fig. 2D that, the best examined antimicrobial agent was that of the mixture of AuNPs and Imtenan® citrus blossom honey (1:1 = v:v) in which the best MBC observed was at the concentration of 0.56×10^9:0.3 (NP/ml: honey %v/v) against MRSA + VSSA (Rows A, B, C & H) followed by 2.24×10^9:1.25 (NP/ml: honey %v/v) against MRSA + VRSA isolates (Rows E, F and G) while the lowest MBC was observed at 4.5×10^9:2.5 (NP/ml: honey %v/v) against MRSA + VRSA isolate (Row D). On the other hand, the best observed MIC was respectively as follows, 0.28×10^9:0.15 (Rows B), 0.56×10^9:0.3 (Rows A, C & H), 1.12×10^9:0.625 (Rows G), 2.24×10^9:1.25 (Rows E & F) and finally at 4.5×10^9:2.5 (Rows D) (NP/ml: honey %v/v) as presented in Fig. 2D and Table 1. These results reporting that the mixture of the Imtenan® Citrus Blossom honey with the AuNPs nanoparticles is a new collective phenomena give rise to a novel approach against MRSA and VRSA isolates implicated in the mastitis cases.

5. Conclusions

In conclusion, this study demonstrated high prevalence and counts of *S. aureus* in the mastitis milk samples examined, and MRSA and VRSA were found to be implicated in a large extend in theses mastitis cases. We recommended that the Pre-milking udder preparation, the sanitization of workers' hands before milking, the quick investigation of the clinical and subclinical mastitis with quick determination of their causative agent, the restricted separation between the infected and healthy animal, the prevention of the misuse or extensive therapeutic use of antibiotics like penicillin or closely related antibiotics, and the antimicrobial susceptibility testing before any drug administration, were very important critical precautions in order to prevent the drug-resistance MRSA or VRSA developments or contagious spreading. Furthermore, any infected animal refused the treatment, the culling of that animal will be the final choice to prevent MRSA and VRSA contagious spreading. Moreover, the present in vitro experiment concluded that the authorized Imtenan® citrus blossom honey mixed with AuNPs have crucial in vitro antimicrobial activities against MRSA and VRSA. Further studies are now required from us to characterize the antimicrobial components in both antimicrobial agents to demonstrate which mechanism facilitates their antimicrobial effect with required in vivo clinical applications.

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