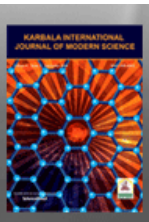

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Controlling the heterogeneous vancomycin intermediated Staphylococcus aureus (hVISA) through the use of Rosmarinus officinalis L. leaves extract

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Keywords

antibacterial activity, antimicrobial activity, chemical composition, real-time PCR

RESEARCH PAPER

Controlling the Heterogeneous Vancomycin Intermediated *Staphylococcus aureus* (hVISA) Through the Use of *Rosmarinus officinalis* L. Leaves Extract

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Abstract

Treatment failure and persistent infection are two serious problems that result from *Staphylococcus aureus* having decreased vancomycin susceptibility. The present study investigates the effects of *Rosmarinus officinalis* L. leaves extract on the growth and quorum sensing (QS) of heterogeneous vancomycin-intermediate *S. aureus* hVISA isolates. Real-time RT-polymerase chain reaction (PCR) is used to determine the transcriptional changes of the accessory gene regulator (*agr*) in the (hVISA) strain after and before being treated with the *R. officinalis* leaves extract. The antibacterial activity of the EOs was determined through the use of a microtitre plate test (MtP); with a minimum inhibitory concentration (MIC) of 0.7 mg/ml against hVISA, a rosemary leaves extract has a high level of inhibitory activity. However, the extraction of essential oils is done in a soxhlet apparatus by using ethanol 96%. Gas chromatography and mass spectrometry (GC/MS) and revealed the most common chemicals containing verbenone, 36.20%, and 1,8-cineol (Eucalyptol), 12.14%. Finally, after treatment with sub-MIC concentration, *hld* gene levels significantly decreased in hVISA strains (a mean of 0.4-fold) in comparison to the control strains (1.0-fold) in late-exponential phases, regardless of whether the *agr* mutation is present. The down regulation of the *hld* gene may be a significant genetic event in the hVISA strain.

Keywords: Antibacterial activity, Antimicrobial activity, Chemical composition, Real-time PCR

1. Introduction

Staphylococcus aureus is a major human pathogen that causes skin and soft tissue infections and life-threatening systemic diseases. It is associated with a high rate of morbidity and mortality worldwide [1]. *S. aureus* is well adapted to various environments due to its metabolic adaptability and pharmic resistance. *S. aureus* colonizes the skin and nasopharyngeal membranes of about 25−30% of healthy people. It coexists with other members of the normal microbiota and doesn't infect people with a healthy immune system [2]. However, *S. aureus* can cause various dangerous diseases if it spreads through more severe infections, such as endocarditis or osteomyelitis, or enters the bloodstream or other internal tissues [3].

Glycopeptide group antibiotics, especially vancomycin, have been the mainstay of the conventional treatment of infections that are caused by methicillin-resistant *S. aureus* (MRSA) [4]. The use of Vancomycin has increased due to the rise in MRSA infections, but *S. aureus* strains that are less susceptible to vancomycin have also emerged. Terms such as "vancomycin-intermediate *S. aureus* (VISA)" and "heterogeneous vancomycin-intermediate *S. aureus* (hVISA)" have been used in scientific literature [5].

In several parts of the world, low-level vancomycin resistance in *S. aureus* (hVISA/VISA) has become a significant clinical problem [4,6,7]. hVISA and

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VISA strains have been linked to severe infections that result in glycopeptide treatment failure [8,9]. hVISA (vancomycin MIC, ≤2 μg/ml) but containing resistant subpopulations of cells are able to grow in the vancomycin-intermediate range (VISA, MIC 4–8 μg/ml) [7,10]. VISA strains with vancomycin MICs of 4–8 μg/ml by broth dilution have received much attention [9].

The Infections that are caused by hVISA strains pose unique challenges in hospitals. Studies [11,12] found that patients with hVISA had a challenging clinical course and high rate of infection-attributable mortality. Infections with hVISA are associated with a greater chance of vancomycin treatment failure, prolonged hospital admissions, a higher risk of persistent disease, and higher treatment costs [8].

Antibiotic-resistant *S. aureus* has defeated the opinion of the antibiotic trust. Health professionals and researchers worldwide have been interested in aromatic and therapeutic plants in response to such resistance [13], which have antibacterial characteristics due to the amount of essential oil they have [14].

The development of novel drugs and improved human health and wellbeing have greatly benefited from the natural products, including medicinal plants. It has also been noted that various organic compounds and plant extracts can stop the growth of germs like *S. aureus* [16]. They can serve as helpful natural models for creating novel medications or phytomedicines that have been purified to treat the disease [15]. However, there are few molecular biology studies about this case.

Rosmarinus officinalis L., also known as rosemary, is widely used in folk medicine, pharmaceuticals, and cosmetics. It belongs to the Lamiaceae family [17], which includes over 230 genera and more than 7000 species [18]. The leaves have been widely used in Iraq for several years to treat a variety of diseases. It is desirable due to its medicinal benefits and bioactivity [19]. Such benefits include anti-inflammatory [20], anti-diabetic [21], liver-protecting [22], and antimicrobial [23] properties. The current study discusses the chemical makeup and antimicrobial activity of *R. officinalis* leaves extract against clinical isolates of hVISA by estimating the inhibitory effect and measuring the expression level of the *hld* gene of the hVISA strain before treatment and after.

2. Material and methods

2.1. Plant material and collection

R. officinalis L. leaves (Fig. 1) were collected in July 2021 from the Al-Amriyah neighborhood in Almansour district of western Baghdad. The plants were classified at Mustansiriyah University, College of Science, Department of Biology, in Baghdad, Iraq. The leaves were washed with water, allowed to dry at room temperature in the shades for two weeks, and then ground with an electric grinder into particles of no more than 1 mm. The ground leaves were then stored at 4 °C in the refrigerator before being used to extract the essential oil from them.

Fig. 1. Rosmarinus officinalis L. *leaves.*

2.2. Chemicals and reagents

The following chemicals and reagents were brought from the following places: Pure ethanol from Scharlab of Spain. 2, 3, 5, Triphenyl tetrazolium chloride from Himedia of India. Dimethyl sulfoxide (DMSO), from Thomas Baker, India., the Vancomycin Powder and Glycerol from RPI Company in USA, a standard turbidity solution (McFarland No. 0.5) from BHD of England and the TRIzolTM reagent from Thermo Scientific USA.

2.3. Preparation of extracts

The ethanolic extract of *R. officinalis* L. leaves that is used for this study is prepared according to Kőszegi et al. [24], with some modifications to the original technique. Twenty grams of dried ground plant material were placed in thimbles of Soxhlet apparatus with 250 ml of 96% ethanol and extracted for 12 hours at 60–70 °C to get a sufficient amount of the extract. The process is repeated several times. The extract is then put in a rotary evaporator and kept in the refrigerator at 4 °C.

2.4. Performance calculations

The yield is defined as the ratio between the mass of the leaves extract that is obtained after extraction

(M′) and the mass of the plant material that is used (M), according to AFNOR (1986) [25] standard. The yield is as follows:

$$RHE\ (\%) = (M'/M) \times 100 \quad (1)$$

RHE (%): Yield of the leaves extract.
M': M: mass of leaves extract.
M: M: mass of dry plant material.

2.5. Chemicals containing the Rosmarinus officinalis L. leaves extract

In the Corporation for Research and Industrial Development of Ministry of Industry and Minerals in Baghdad, Iraq, GC-Mass is used with an Agilent 7820 Gas Chromatography (Agilent Technologies, Wokingham, United States), coupled with an Agilent 5977 MSD and an Agilent HP-5MS Ultra Inert column (30 m long, 250 m wide, 0.25 m inside diameter) to analyze the leaf extract of *R. officinalis* L. Injection is performed with a splitless technique. The temperature of column's oven was set first to 50 °C for 1 min before being increased to 150 °C with an 8 °C/min heating ramp, and then from 150 °C to 280 °C at an 8 °C/min heating ramp at a terminal temperature of 280 °C per 3 min. The spectra were in electronic impact mode with ionization energy of 70 eV. The volume of the injected leaves extract sample is 1 μL. The carrier gas is helium, with a 1 mL/min flow rate. The total ion chromatography uses computerized literature and MS data from the NIST11 library [26] to figure out the GC–MS relative percentages of the isolated compounds.

2.6. Collection of S. aureus isolates

The study obtained 89 non-duplicate *S. aureus* isolates from different clinical specimens, including wounds, urine, burns, stool, blood, exudates, and respiratory samples during a period from February 2021 to January 2022. Patients in Al-Yarmouk Teaching Hospital, Central Pediatric Teaching Hospital, and Al-Karama Hospital were included in this study. These isolates were stocked in 20% glycerol broth and kept at −20 °C until they are to be used for further analysis.

2.7. Identification of S. aureus isolates

The mannitol salt agar and brain heart agar were used to isolate *S. aureus,* and probable colonies were picked and isolated for additional diagnostic tests based on morphological characteristics. *S. aureus* was identified using Bergey's manual of systematic bacteriology [27].

2.8. Methicillin resistance S. aureus identification

Isolates that were identified as *S. aureus* were cultured on CHROMagar™ MRSA medium. The medium was used as a selective medium for MRSA. The appearance of positive colonies ranges from rose to mauve. Other identification methods have included 16SrRNA [28] and molecular PCR [29]. These isolates were stored in a deep freezer in glycerol vials.

2.9. Detection of hVISA/VISA by method of screening

The detection of hVISA/VISA in all MRSA was performed by the use of Brain Heart Infusion Agar (BHIA) with six μg/ml vancomycin [30]. 10 μL of 0.5 McFarland suspensions of the strains were spot inoculated in BHIA with six μg of vancomycin. The plates were incubated for 24 hours at 35 °C ± 2 °C and checked for any single colony of *S. aureus* strain growth.

2.10. Standard vancomycin Etest

The standard vancomycin Etest (BioMerieux, Marcy l'Etoile, France) was performed and interpreted according to the manufacturer's directions. A macro-method E test was performed on overnight cultures of the original isolates to detect possible hVISA. The suspensions in BHI broth were adjusted to a 0.5 McFarland standard, and samples were swabbed onto Mueller–Hinton agar (MHA) with 5% sheep blood. The intersection of hazy development or microcolonies is read after inhibition is completed. After 48 hours at 35 °C, the intersections of the elliptical recorded a growth in the inhibition zone and in the inhibition zone and the development of detectable hVISA. Isolates with vancomycin values of ≥8 μg/ml at 48 hours were considered as h-VISA [31–33].

2.11. Confirmatory test population analysis profile-area under the curve (PAP-AUC)

The PAP-AUC method, which is still the gold standard for confirming hVISA/VISA isolates, was used to test *S. aureus* strains for hVISA/VISA. Standard strains of Mu3 (ATCC 700698), Mu 50 (ATCC 700699), and *S. aureus* (ATCC 29213) were used. In brief, overnight *S. aureus* broth cultures were diluted to get 10^3, 10^6, 10^8 and inoculated on BHIA containing vancomycin concentrations of 0.5 μg/mL, 1.0 μg/mL, 2 μg/mL, 2.5 μg/ml, 4 μg/mL, 6 μg/mL, 8 μg/mL and 16 μg/mL. After 48 hours of incubation

at 37 °C, the colonies in each plate were counted and plotted against the corresponding vancomycin concentration by the use of GraphPad Prism 9.3.0 (GraphPad Software Inc., San Diego, USA). To calculate the AUC and distinguish between VSSA, hVISA, and VISA, AUC Ratio test strain to Mu3 (ATCC 700698) was obtained. A ratio of ≥0.9–1.3 was used to diagnose hVISA and >1.3 for VISA [34].

2.12. Determination of minimum inhibition concentration (MIC)

MIC of the present study was determined by the use of techniques of Abu-Shanab and Abou Elkhair [35,36]. The Mueller–Hinton broth was autoclaved (20 minutes at 121 °C) and then cooled to 45 °C for microorganisms to be distributed by 50 µl into each well of the 96-well plates. Then, a 50 µl extract stock solution (200 mg/ml) was filled into the first row of the well; a micropipette was used to carry out two-fold serial dilutions; the range of concentrations was 200 to 0.39 mg/ml.

Except for the positive control, 10 µl of hVISA strain was put into each well (inocula were adjusted to 0.5 McFarland) and was used to employ positive control of plant extract with the medium and negative control of inoculum with the medium.

The test plates were incubated for 18 hours at 37 °C. Afterwards, the well plates were filled with 50 µl of a 0.01% solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC) and incubated for another hour.

The biological activity of the bacteria reduced the colourless tetrazolium salt to red-coloured result. Growth is suppressed when the well's solution remains clear after incubation with TTC. The MIC was the lowest sample concentration that stopped the growth and didn't change colour, (so it was clear).

2.13. Total RNA extraction

Total RNA was extracted from heterogeneous vancomycin-intermediate *S. aureus* (hVISA) samples according to the protocol of TRIzol™ Reagent (Thermo Scientific, USA).

2.14. Quantitative real-time PCR (qRT-PCR). One-step real-time qRT-PCR

A delta-hemolysin gene (*hld* gene) expression was detected by the use of quantitative real-time PCR (qRT-PCR). 16srRNA Housekeeping gene was used for calibration and the primers were prepared according to the directions of the supplying company.

The gene expression was measured in the hVISA before and after treatment with *R. officinalis* leaf extract. The sub-MIC value of *R. officinalis* leaf extract was used to test gene expression.

The manufacturer's instructions were followed about the use of a one-Step RNA PCR Kit (GoTaq® 1-Step RT-qPCR System, MgCL2, and Nuclease Free Water); the primers are: 5′-ATTTGTTCA-CTGTGTCGATAATC-3′ and 5′-GGAGT GATTT-CAATGGCACAAG-3′ are the forward and reverse primers, respectively [37] (16srRNA): FP 5′-CTG-CTGCCT CCCGTAG-3′ and RP 5′-CCGACCTGA-GAGGGTGA-3` [38].

Volumes of each single reaction were ten µL reaction mix containing five µL qPCR Master Mix, 0.25 µL RT mix, 0.25 µL MgCl2, 2.5 µL Nuclease Free Water, 0.5 µL FP, 0.5 µL RP (Macrogen, Korea) and one µL RNA.

The following reaction is to perform a DNA amplification: One cycle for RT. Enzyme activation at 37 °C for 15 minutes, followed by 5 minutes of initial denaturation at 95 °C. Denaturation at 95 °C for 20 seconds, annealing at 55 °C for 20 seconds, and extension at 72 °C for 20 seconds are alternated over 40 cycles. The expression levels of the gene of interest were calculated by the use of the cycle threshold number (CT) value [39], and gene expression of RNAIII (*hld* gene) was quantified by the use of 16s rRNA as a housekeeping gene.

2.15. Statistical analysis

The collected data were subjected to an analysis of variance (ANOVA) for a completely randomized design. The Least Significant Difference (LSD) test was used to compare the means that differed statistically significant at p 0.05. The statistical analysis was carried out by the use of GenStat 17th Edition (VSN International Ltd., Hemel Hempstead, UK) [95].

3. Results and discussion

3.1. Income and organoleptic characteristics

R. officinalis L. leaf extract is a viscous dark brown liquid that smells aromatic. The extract yields 18%, which is higher in comparison to Jordan's 12% [40], China's (16%) [41], and Morocco's Taounate region (17%) [42]. Tavassoli and Djomeh in Iran reported a greater yield of 18.9% [43].

Environmental factors like sun exposure have an impact on the morphological parameters and extraction yield [44]. Drought stress can also impact the production of essential oils positively due to the water deficit that is brought on by temperature and solar radiation. Temperature has been shown to

affect the activity of extraction yields significantly [45].

According to Gershenzon et al. [46], water shortages may contribute to higher density and greater essential oil content of leaves that were produced under water stress that illustrates a physiological mechanism and explain why essential oils accumulate more under stress. Rather than directly influencing stress on secondary metabolism, the increased amounts of essential oils seem to result from growth and development changes. [47].

3.2. Chemical composition of the leaves extract
Rosmarinus officinalis L

Gas chromatography–mass spectrometry was used to analyze the *R. officinalis* leaf extract. The primary components of the leaves extract was identified (Table 1): Verbenone, 1,8-cineole (Eucalyptol), and Benz (a) acridine of 9,10,12-trimethyl with values of 36.20%, 12.14%, and 9.68%, respectively, Borneol of (9.39%), 2-(3,3dimethylcyclohexylidene)-(Z)-(ethanol)of (8.63%), camphor of (3.07%), 5-Androstene of (4,4-dimethyl), of (2.22%), and caryophyllene of (2.14%).

R. officinalis L. extract has been the subject of several investigations worldwide. Factors of climate, seasons, geography, and genetic variations affect the essential oil structure [48,49].

As a result, this study was consistent with the prior investigations, suggesting that verbenone was the primary component of *R. officinalis* extract in Iran [50], Italy [51], and Spain [52], but was not detected in other studies that were conducted in Iran [53], Tunisia [54], Morocco [55], and Serbia [56].

The second most important chemical in *R. officinalis* extract is 1,8-cineole (Eucalyptol). This chemical is also a major part of the rosemary extract, which has been studied before [57].

The principal elements in this study investigation were verbenone and eucalyptol, which were found to be substantial in an Iraqi study done in Erbil; Ahamad et al. [58] discovered the primary ingredients in their extract: verbenone of (23.46%), 1,8-cineol (Eucalyptol) of (15.96%), α-pinene of (12.10%), camphor of (10.98%), and bornyl acetate of (5.78%).

A recent study done by Hamid [59] harvested rosemary samples in October 2017 from Diyala province that is located at the eastern side of Iraq, and found out that the critical components in rosemary samples extracts are as follows: 1,8-cineol (Eucalyptol) of (59.02%), camphor of (29.09%), α-Terpineol of (3.75%) and Bornyl acetate of (2.83%).

Table 1. The chemical complex of R.officinalis *leaves extract.*

Retention time	Constituents	Classification	Chemical formula	Molecular weight	Area %
6.245	1,8-cineol (Eucalyptol)	Oxane	C10H18O	154.25	**12.14**
7.887	Linalool	monoterpenoid	C10H18O	154.25	0.77
8.417	Camphor	monoterpenoid	C10H16O	152.23	3.07
9.092	Borneol	monoterpenoid	C10H18O	154.25	9.39
9.776	Verbenone	monoterpenoid	C10H14O	150.22	**36.20**
10.776	Ethanol,2-(3,3-dimethylcyclohexylidene)-, (Z)-	Pheromones	C10H18O	154.25	8.63
11.717	Farnesyl acetate	Sesquiterpenoids	C17H28O2	264.4	0.84
12.554	6-Methylspiro[4.5]decan-6-ol	Tertiary alcohols	C11H20O	168.28	0.84
13.119	beta-Caryophyllene	Sesquiterpenoids	C15H24	204.35	2.14
13.717	4-Isopropyl-cis-bicyclo[4.3.0]-2-nonen-8-one, (4R,S)-	Nf.	C12H18O	178.27	0.65
16.880	Decahydro-1-naphthol	Cyclic alcohols and derivatives	C10H18O	154.25	0.71
19.017	cis-Pinane	Monoterpenoids	C10H18	138.25	0.65
20.231	Hexadecanoic acid, methyl ester	Fatty acid esters			1.26
22.360	(R)-(−)-14-Methyl-8-hexadecyn-1-ol	Fatty alcohols	C17H32O	252.4	2.09
23.121	1-Monolinolenoyl-rac-glycerol	Linoleic acids and derivatives	C21H36O4	352.5	0.72
25.394	1-Hexanesulfonanilide,4'-(3-acetamido-9 acridinylamino)-	Nf.	C27H30N4O3S	490.6	0.74
25.694	1,3,5-Tris(trimethylsiloxy) benzene	Phenoxy compounds	C15H30O3Si3	342.65	1.97
26.617	1-(4-Methyl-[1,1':4',1"]terphenyl-4″-yl) ethan one	Nf.	$C_{15}H_{14}O$	210.27	1.82
26.771	5-Androstene, 4,4-dimethyl-	Nf.	$C_{21}H_{34}$	286.49	2.22
26.814	Ferruginol	Diterpenoid	C20H30O	286.5	1.51
27.113	Benz(a)acridine, 9,10,12-trimethyl	NF.	C20H17N	271.4	**9.68**
28.010	Dehydroabietinol	Isoprenoids	C20H30O	286.45	0.75
29.583	Squalene	Triterpenoids	C30H50	410.7	1.20

Bold values show the bulk of the leaves extract.

3.3. Prevalence of hVISA strains by method of screening and confirmatory

Among 89 *S. aureus* isolates were identified by following standard microbiological procedures, 30 MRSA strains have an isolated rate of 33.7%. They were grown on CHROMagar™ MRSA medium and confirmed by the *mecA* gene. In Etest, 2 of (6.6%) from all MRSA isolates in this study were positive for hVISA (Fig. 2); MIC for the positive isolate was ≥8 μg/ml. Chaudhari et al. [60] observed that the prevalence of hVISA was of 6.9% with PAP-AUC and the Etest macro method. Walsh et al. [31] developed the Etest macro method with breakpoints of vancomycin 8 μg/mL, which was sensitive and specific for h-VISA confirmation but was too costly for routine screening.

Two of (6.6%) MRSA isolates were hVISA by the vancomycin screening. This test was done before the PAP-AUC, as it was done by a study in Turkey; Sancak et al. [61] looked at 256 clinical MRSA isolates from 256 individuals and discovered 46 of (17.97%) hetero VISA isolates by the use of the PAP-AUC technique after screening with BHI agar containing vancomycin.

The present study confirms only 1 of (3.3%) as hVISA, three strains of (10%) as VISA, and one as VSSA by the PAP-AUC techniques (Figs. 3 and 4). The prevalence rate of hVISA is almost consistent with that of a study done by Iyer and Hittinahalli of India that showed an hVISA prevalence rate of 2% [62].

In the case of Japan, hVISA is shown to be of 3% (34/1149 MRSA) [63]. In Italy, Germany, France, and the Netherlands, the prevalence of hVISA in MRSA was of 1.1%, 0.21%, 0.6%, and 6%, respectively [64–67]. Thus, most hVISA differs slightly among different countries. Nevertheless, some actual differences among the countries do occur due to detection methods.

3.4. Antibacterial activity

Antibiotic-resistant bacteria are becoming more prevalent and spreading quickly, posing a severe threat to human health due to the rise of multi-drug-resistant (MDR) pathogens, uneven release of new antimicrobial agents, and the effect of antibiotics in treating the infections. Moreover, the rate of development of antibiotics was not related to antibiotic

Fig. 2. A hVISA strain on MHA +5% blood culture yielded a positive E-test macromethod result. Left: Visualize hazy growth and microcolonies, intersections of elliptical inhibition zones, and subpopulation growth in the inhibition zone: Right: an isolated sample of hVISA strain used as reference (Mu3, ATCC 700698).

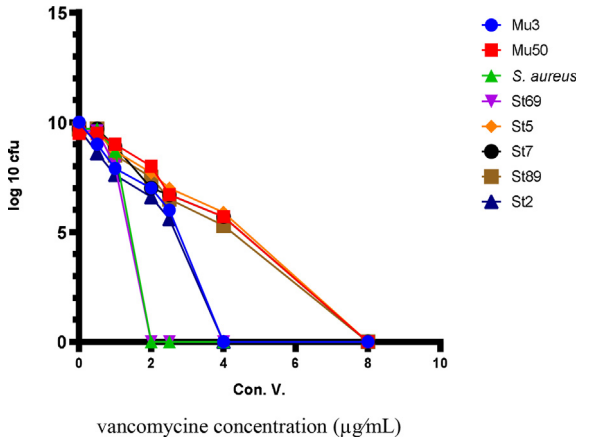

Fig. 3. Population analysis profile curves, one hVISA positive isolate. Three isolates were VISA, and one was vancomycin-susceptible S. aureus *(VSSA) compared to the Mu3 reference strain.*

resistance, which has led scientists to look for new compounds with antimicrobial activity, including medicinal plants in different places [68,69]. Alternative antibiotic medicines can play a role in decreasing the demand for antibiotics and minimizing the emergence of drug resistance by preventing and treating the infectious illnesses [70,71].

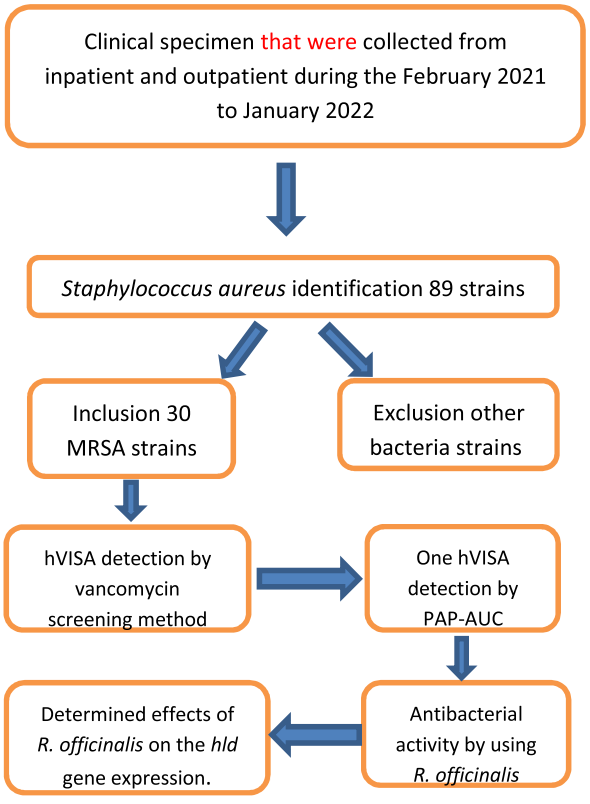

Fig. 4. The steps for confirmation of one hVISA from 89 clinical isolates with R. officinalis *effect steps.*

Pure compounds from natural sources are becoming increasingly supplementary and alternative therapies for various ailments. Several studies proved that *R. officinalis* extract decreases the possibility of bacterial resistance to multiple medicines development [70], therefore, Scientists looked for new compounds in *R. officinalis* that could be used instead of antibiotics.

MIC assays were used in this study to test the extracts of *R. officinalis* leaves to stop the growth of heterogeneous vancomycin-intermediate *S. aureus* bacteria. The results proved that MIC for hVISA isolate was 0.7 mg/mL (Table 2). It was enough to stop the growth of hVISA. The plant extract's MIC against hVISA proved a significant antibacterial activity (p-value = 0.01). This result is similar to Jarrar et al. [72], who discovered a lower inhibitory concentration of 0.39–3.13 mg/ml for MRSA. The result is lower than that of Mekonnen et al. [73], who found that *R. officinalis* leaves have an inhibitory concentration of *S. aureus* at 23.25 mg/ml doses. Furthermore, Golshani et al. [74] investigated the antimicrobial activity of rosemary leaf extract that were brought from Isfahan Region of Iran. They found that it is effective against all strains (*S. aureus, Escherichia coli, Bacillus cereus, and Pseudomonas aeruginosa*). The inhibitory concentration of the extract for these bacteria's growth is from 6.25 mg/ml to 100 mg/ml.

The variation in performance of E. O is due to certain factors, such as the plant origin [75], extraction technique [76], collection period [77], plant material, and ecological and geographical conditions [78].

The Chemical composition, particularly the bicyclic monoterpenes such as Verbenone and Borneol, is responsible for differences in susceptibility. Furthermore, their antibacterial properties are well known [79]. Eucalyptol, also known as 1,8-cineole, is an oxyane that is used to treat sinusitis and bronchitis symptoms because of its antibacterial and spasmolytic properties [80]. It is necessary to note that the synergistic effect of all the *R. officinalis* leaf extract compositions is likely caused by the antibacterial effect of the rosemary leaf extract.

The Bicyclic monoterpenes and oxanoid compounds are antimicrobial because they fight the

Table 2. The minimum inhibitory concentration (MIC) of the leaves extract of Rosmarinus officinalis L.

	MIC(mg/ml)									
Bacteria	200	100	50	25	12.5	6.25	3.12	1.5	0.7	0.3
hVISA ST1	–	–	–	–	–	–	–	–	–	+

-: Inhibition; +: Growth.

cellular enzymes. It depends on how fast the compounds can get into the cell or how well they change the permeability of the cell membrane [81,82].

3.5. Expression of hld genes

Staphylococci have enterotoxins, toxins, hemolysins, and leukocidin that cause tissue damage and immune system evasion [83]. Another essential element in ensuring Staphylococcus spp. survival and protection against the environment is the production of biofilm [84]. There has been a persistent interest in the incredibly huge number of toxins and other virulence factors that *S. aureus* produces and their impact on diseases [85]. However, some scientists have proposed specific underlying causes to explain the connection between antibiotic resistance and the production of hemolysin in *S. aureus* MRSA strains, including its effects on virulence gene expression [86]. Schroeder et al. [87] found that antibiotic resistance genes in *S. aureus* and staphylococcal infections are strongly linked to virulence factors. It means that such factors have a huge impact on the development of antibiotic resistance.

When cell density exceeds a specific threshold, a quorum-sensing system known as Agr (accessory gene regulator) up-regulates several toxins and virulence factors. The staphylococcal virulence regulator has received the most attention by the research [88]. The *agr* locus is among the global regulons that regulates the overexpression and down expression of the most virulence factors [89].

The Agr system effector is a transcript of the P3 operon designated RNAIII which contains 26-amino-acid delta-hemolysin gene (*hld*), the synthesis of which is dependent on the activation of the Agr system and driven by the promoter of the Agr P3 system [90,91].

AgrA activation promotes the transcription of the δ-lysin gene (*hld*) that directly upstream of the Agr operon [90]. RNAIII expression and Agr activity are distinguished indicators of RNAIII expression. The *hld* gene is required for virulence and plays a role in the multiple aspects of staphylococcal pathogenesis [92]. A study of three therapeutic herbs (*Ballota nigra, Castanea sativa*, and *Sambucus ebulus*) found that delta-hemolysin production was dose-dependent; proving the substantial anti-quorum sensing activity in pathogenic MRSA isolates [93]. The current study used the expression of the *hld* gene in an hVISA strain as a functional marker *agr*.

In this study, quantitative RT-PCR measured the mRNA expression (*hld* gene) that is responsible for the production of delta-hemolysin with the Housekeeping gene (H.K) as a calibration [94] by comparing the hVISA growth before and after treatment with *R. officinalis* leaf extract. The amplification was recorded by the use of the Ct values of genes from the quantitative RT- PCR program. The high Ct values show low gene expression, while the low ones show increased gene expression.

Table 3. Genetic expression values for hld gene after and before treatment.

Sample	16srRNA	*hld*	DCT	DDCT	Folding
hVISA	10.60	20.78	10.19	0.00	1.0
hVISA Treated	10.98	22.54	11.56	1.37	0.4

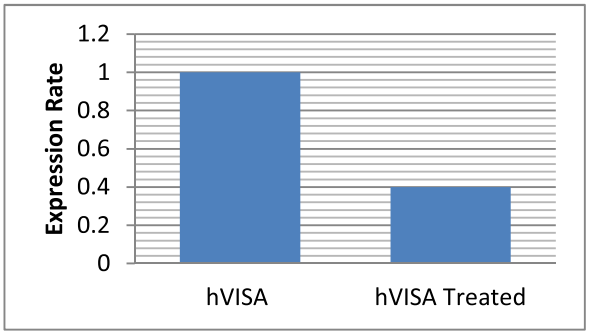

Fig. 5. The Effect of rosemary leaf extracts on hVISA strain, relative expression ratios (gene-to-action ratios) of the (hld gene) gene that codes for delta hemolysin, and the housekeeping gene 16srRNA as a normalized transcription of hld by the comparative CT method.

The results show a decrease in gene expression after being treated with *R. officinalis* leaves extract. The gene expression level was normalized to a housekeeping gene and measured by the use of ΔCT, ΔΔCt value and folding ($2^{-\Delta\Delta CT}$) methods. Table 3 and Fig. 5 show the expression of the *hld* gene according to the treatment with *R. officinalis* leaf extract. The mean Ct value of *hld* gene amplification was (20.78) in control. The Ct value treatment was a mean of (22.54). A significant difference in the mean of increased Ct values indicates low gene expression. Expression of the *hld* gene was low after the treatment.

The results of this study prove that *hld* gene expression is a valuable tool for identifying hVISA resistance programs. The *hld* gene was less active after being treated with an extract of *R. officinalis* leaves at a concentration of 0.7 mg/ml it proved that more researches are needed to determine how other plant extracts affect the *hld* gene.

4. Conclusion

The production of leaves extract was 18%. The most common chemicals are revealed by gas chromatography that is combined with mass spectrometry (GC/MS), which include Verbenone of (36.20%), 1,8-cineol (Eucalyptol) of (12.14%), and

Benz(a) acridine, 9,10,12-trimethyl of (9.68%) as an important constituent. Rosemary leaves extract has a high inhibitory activity with a minimum inhibition concentration of (0.7 mg/ml) against heterogeneous vancomycin-intermediate *S. aureus* (hVISA). The *hld* gene in the hVISA strain decreased after treatment with rosemary leaves extract according to the control. The research supports the idea that *R. officinalis* leaves extract is a better natural source of antibacterial activity than synthetic, i. e it could be used as an antimicrobial agent in industrial productions such as pharmaceuticals.

Conflicts of interest

There are no conflicts of interest regarding the publication of this work.

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