# ERADICATION OF HETEROGENEOUS VANCOMYCIN ITERMEDIATED *STAPHYLOCOCCUS AUREUS* (hVISA) USING *PEGANUM HARMALA* L. SEEDS EXTRACTS

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## ABSTRACT

**The main purpose of this research is to evaluate the antibacterial essential oil of *Peganum harmala* L. seeds against locally isolated heterogeneous vancomycin intermediated *Staphylococcus aureus* (hVISA) at doses ranging from 0.03 to 200 mg/ml. The filtered essential oil of *P. harmala*, after extract by the 96% ethanol in a Soxhlet extraction device, was analyzed by GC/MS (gas chromatography-mass spectroscopy). The results showed that the *P. harmala* seeds oils contained several compounds, such as Harmine (58.26%), followed by 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (28.55%) and Hexadecanoic acid, methyl ester (4.30%), It is highly effective against targeted bacteria. The MIC method was used to investigate antibacterial activity by measuring the lowest inhibitory concentration of the extract and observed that the inhibition concentration against hVISA isolates was 0.3 mg/ml. The gene expression was detected after RNA extract from hVISA (untreated and treated) with *P. harmala* essential oil using the Quantitative Real-Time PCR method the results revealed that gene expression was low after bacterial with plant extract treatment. Finally, the ethanolic extract of *p. harmala* seeds appears to be a potential therapy choice for hVISA infection.**

**Keywords: Antimicrobial activity, antibacterial activity, chemical composition, essential oil.**



# القضاء على على المكورات العنقودية الذهبية غير المتجانسة (hVISA) *S. AUREUS* باستعمال مستخلص بذور الحرمل *P. HARMALA* L.

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## المستخلص

**الغرض الرئيس من هذا البحث هو تقييم الزيت الاساسي لبذور نبات الحرمل المضاد للبكتريا ضد المكورات العنقودية الذهبية غير المتجانسة متغايرة المقاومة تجاه الفانكومايسين بجرعات تتراوح من 0.03 الى 200 ملغم/مل , تم تحليل الزيت الاساسي المفلتر بعد استخلاصه من 96% ايثانول في جهاز الاستخلاص سوكسلت بواسطة الكروماتوغرافيا الغازية / المطياف الكتلي , اظهرت النتائج احتواء زيوت بذور الحرمل على عدة مركبات منها Harmine (58.26%) ثم methyl ester, (ZZ) 9,12-Octadecadienoic acid (28.55%) و Hexadecanoic acid, methyl ester (4.30%) وهي فعالة للغاية ضد البكتريا المستهدفة, تم استخدام طريقة التركيز المثبط الادنى لفحص النشاط المضاد للبكتريا عن طريق قياس اقل تركيز مثبط للمستخلص ولوحظ ان تركيز التثبيط ضد العزلات كان 0.3 ملغم/مل , تم الكشف عن التعبير الجيني بعد استخلاص الحمض النووي الريبي من العزلة البكتيرية المعاملة والغير معاملة بالزيت الاساسي لنبات الحرمل, اخيرا يبدو ان المستخلص الايثانولي لبذور نبات الحرمل علاج محتمل لعدوى المكورات العنقودية الذهبية غير المتجانسة**

**الكلمات المفتاحية: الفعالية المضادة للميكروبات, الفعالية المضادة للبكتريا, المكونات الكيميائية, الزيوت الاساسية.**

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## INTROUCTION

Medicinal plants and herbs have been regarded as critical medicinal products. Muntaha and Majid (30) observed that the disinfection product (Dettol) contains a herbal formula. They also compared the efficacy of this formula with that of traditional Dettol, and they discovered that the composition of a herbal disinfectant and antiseptic was similar to that of industrial Dettol. Ventola (47) reported that many medicinal plants are used in traditional medicine as alternative medicines due to their numerous applications in cosmetic pharmaceutical, food, and other industries, which have attracted much attention (33). Harmala is a perennial herbaceous plant in the Nitrariaceae family, mainly found in dry Mediterranean and Asian regions. This plant has therapeutic and ecological importance (54). *P. harmala*, also known as Syrian Rue, is a medicinal herb found in North-West India, North Africa, and Central Asia semi-arid areas. This plant is known in Iran as "Espand," in North Africa as "Harmel," and in the United States as "African Rue," "Mexican Rue," or "Turkish Rue" (24); Bushy wildflowers reaching 60-90 cm in height with a short creeping root (24). Harmal species have shown antimicrobial properties against various microorganisms (36,44), killing or inhibit the microbes that cause disease. For this purpose, several antimicrobial agents are used, such as antibacterial, antiviral, or antifungal compounds. They all have different modes of action to suppress infection (36,45). *Staphylococcus aureusis* agram positive bacteria that growsin the form of clusters with 0.5 to 1.5µm in diameter portrayed by individual cocci since division of cell happens in more than one plane, these bacteria are non-motile, non-spore forming (13) *Staphylococcus aureus* is a bacterium found in the skin and nasal membranes that have the potential to cause a wide range of community and hospital-acquired illnesses (53) Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) is linked to vancomycin treatment failure and is becoming a public health issue (55). Furthermore, study (26) linked the hVISA phenotype to deep endovascular infections, such as infective endocarditis (IE). The accessory gene regulator (*agr*) in *S. aureus*, is a crucial virulence regulator. RNAIII is the system's effector, known for up-regulated toxin expression and down-regulated genes encoding cell surface-associated proteins (32). The *agr* operon is composed of 2 divergent promoters, P2 and P3, regulated operon produces the *agr* effector molecule RNAIII, which is responsible for the post-transcriptional regulation of numerous virulence factors, including α-hemolysin and δ-hemolysin (both encoded within the RNAIII transcript) (16,52). The absence of δ-hemolysin expression was most likely the source of *agr* deficiency (38). The divergent *agr* transcript, stimulated by P3, produces RNA molecular (RNAIII), which overlaps the *hld* gene and encodes delta-hemolysin (32). Delta-hemolysin is unusual among the secreted virulence factors regulated by *agr* since it is encoded by *hld* inside the *agr* locus and produced by RNAIII translation (31). Used Delta-hemolysin expression to determine *agr* function in an *S. aureus* strain containing the *hld* gene. Clinicians treating severe infections, especially endovascular infections (e.g., infective endocarditis) caused by the hVISA, have faced significant challenges due to antimicrobial resistance in *Staphylococcus aureus.* Bactericidal therapy is critical for successfully treating these infections (21). This study was examine the effect of *P. harmala* essential oil to determine its chemical composition and antimicrobial activity against clinical isolates of hVISA by estimating the inhibitory action and quantifying the expression level of the *hld* gene of hVISA strain after and before treatment.

## MATERIALS AND METHODS

### Plant of materials collection

The seeds of *Peganum harmala* L. were collected in September (2021) from a local market in Baghdad city (Baghdad / Iraq) and identified by a professional taxonomist in the Department of Biology / College of Science / Mustansiriyah University. The seeds were cleaned with water and left to dry in the shade for two weeks before extracting the essential oil, grinding it with an electric grinder, and storing it in the refrigerator at 4 C.

**Plant extract preparation**

Ethanolic extract of *Peganum harmala* L. seed prepared an according to Kőszegi *et al*. (20), with some modification to the original technique. The ethanolic extract was obtained by putting 50g of *P. harmala* seeds powder with 500 ml of ethanol 96 % in a Soxhlet extraction unit. The extraction continued for the next 16 hours at 45-60°C. Whatman No. 42 (125 mm) filter paper was used to filter seed extract oil. Then, the extract in Vacuum Rotary Evaporator was concentrated at 35°C and stored at 4°C in sterile tubes. 10 ml of Dimethyl sulfoxide (DMSO) was mixed with 1g of dried extract and sterilized using a membrane filter (0.22 m) before antibacterial testing. The percentage of oil in *P. harmala* seeds was Percentage of oil in Oil seeds (%) = (Weight of oil (gm) / Weight of the sample (gm)) χ 100.

**GC/MS analysis**

*P. harmala* seeds were analyzed using a gas chromatography (GC-Mass) spectrophotometer with an Agilent 7820 Gas Chromatography (Agilent Technologies, Wokingham, United States). Coupled to Agilent 5977 MSD, Agilent HP-5MS Ultra Inert column (30m length x 250 µm diameter x 0.25 µm inside diameter). Extracted *P. harmala* seeds were autosampler inside the capillary column. injected with a splitless method with 1µL volume of extracted material. A carrier gas of 99.999 % purity was employed as a carrier gas, with a constant flow rate of 1 ml/min. Temperatures of the injector and detector were set to 50-280°C, programmed oven temperature of the column firstly at 50 °C to 1 min and later expanded to a 150°C with a rate of 8 °C/min heating ramp. Then heating 150 °C to 280 °C at 8 °C/min ramp, at a terminal temperature of 280°C per 3 min. The seed extract was identified as peaks by comparing mass spectra to a mass spectral database (40)

**Collection of bacteria**

About 165 samples, including 58 inpatients and 31 outpatient samples, such as burns, foot ulcers, blood, sputum, pus, wounds, and ear infections was collected from local hospitals in Baghdad (Al-Yarmouk Teaching Hospital, Central Pediatric Teaching Hospital, Al-Karama Hospital, al Furat General Hospital, and Ibn Al-Bitar center for cardiac surgery Hospital). Duration ranged (from February 2021 - to January 2022).

**Bacterial isolation**

Mannitol salt agar and Brain heart agar were used to isolate *S.aureus* The 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 (49).

**Identification of *staphylococcus aureus* isolates:** Gram stain, catalase, oxidase, mannitol ferment, and coagulase testes used to identify the isolates. .**Identification of methicillin resistance *Staphylococcus aureus***

In this study, cultured isolates identified as *Staphylococcus aureus* on a selective medium CHROMagar™ MRSA; the rose to mauve color detected a positive colony. Other identification methods include *mecA* detection using the Master thermocycler gradient PCR to amplify the extracted genomic DNA (Eppendorf, Germany). MRS1 - TAGAATGACTGAACGTCCG; MRS2 -TTG CGATCAATGTCCGTAG are the primers needed to amplify a fragment of *mecA* (18) These primers were provided in lyophilized form, dissolved in sterile nuclease-free water to prepare 100 pmol µl as a final concentration, and stored in the deep freezer until used in PCR amplification manufacturer's instructions. All PCR reaction tubes had a final volume of 25 µl. Five microliters of PCR premix (Taq PCR Master Mix) and one microliter of each primer were included in the reactants, while the DNA template volume was 1.5 µl. After that, sterile nuclease-free water was used to fill the volume up to 25 µl. After that, the mixture was gently vortexed, and the PCR tubes were briefly centrifuged to ensure good mixing. After several trials, the adopted PCR protocol was followed, An initial denaturation for one cycle of 3 minutes at $94^{o}$C was followed by 35 cycles of 30 seconds denaturation at $94^{o}$C, annealing for 30 seconds at $58^{o}$C for 35 cycles, extension for 30 seconds at $72^{o}$C for 35 cycles, and a final extension for 10 minutes at $72^{o}$C for one cycle. In addition, the cefoxitin disk method was used to test for MRSA (18), and the isolates were stored in glycerol vials in a deep freezer.

**Vancomycin screening method**

All MRSA isolates that inoculate on brain heart infusion agar (Liofilchem, Italy) were treated with Vancomycin (Sigma Chemical Co, USA) at a concentration of 4 µg/ml (15) Then add to BHIV4 (brain heart infusion with vancomycin 4 µg/ml) 10µl bacterial suspension of 0.5 McFarland defined hVISA after 48 hours incubation (43); each plate had a negative control of ATCC *S. aureus* 25923 and positive control of ATCC-700698, Mu3 strain of hVISA.

**Standard E-test**

Vancomycin MICs are determined using the E-test, a screening method for hVISA. On Mueller-Hinton agar (MHA) (Oxoid, UK) plates with 5% blood, inoculated 10 µl of 0.5 McFarland of all MRSA plates, using vancomycin E-test strips (BioMerieux, Marcy l'Etoile, France) with incubation 48 hrs. MIC values were evaluated at the actual endpoint, as recommended by the manufacturer for results reporting. The test is positive when the MIC of the vancomycin is ≥8 µg/ml. After inhibition, read to visualize hazy development or microcolonies with care (21, 48).

**Population Analysis profiling area under the curve (PAP-AUC):** This study screened MRSA isolates in (PAP-AUC) using the technique published by Wootton *et al*. (51); BHI broth (Liofilchem, Italy) used to incubate colonies overnight at 37°C as part of this study. prepared the log dilutions in $10^{-3}$, $10^{-6}$, and $10^{-8}$ in 0.9% sterile saline. 100 µl of dilutions were lawn grown on a BHI agar (Liofilchem, Italy) containing vancomycin at 0 to 8 µg/ml doses. Plotted the viable count versus the vancomycin concentration after 48 hrs. of incubation at 37°C and calculated the AUC using colony-forming unit (CFU/ml) values, employing Graph Pad Prism software version 9.3.0. (GraphPad Software Inc., San Diego, USA). used VISA (ATCC-700699, Mu50), *S. aureus* (ATCC-29213), and hVISA (ATCC-700698, Mu3) as control strains. If the test-to-control AUC ratio was ≥0.9 and <1.3, the isolate was considered an hVISA, and if it was ≥1.3, it was considered a VISA.

**Minimum inhibitory concentration of plant extracts:** According to (2,3), this study determined the MIC using a 96-well microtiter plate. filled the first row of the well with 50 µl tested extracts stock solution to prepare concentrations ranged (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 mg/ml) by serial twofold dilutions in 50 µL of Mueller Hinton broth (Oxoid, UK). Each well contained 50 µl of seeds oils and 50 µl of Mueller–Hinton broth. Then, the inocula adjusted to 0.5 McFarland and added 10 µl to each well except for the positive control and incubated plates at 35°C for 18–20 h. The positive control as the plant extract used with media and the negative control for the inoculum with media. After incubation, bacterial growth determined by adding 50 µl of 0.1 % 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution to each well and incubating at room temperature for 45-60 minutes. The minimum inhibitory concentration (MIC) determined as the lowest concentration of plant extract. After incubation, growth reduced the tetrazolium dye to a red/pink; detected growth inhibition was when the solution in the well remained clear after incubation with TTC.

**Gene expression analysis**

A delta-hemolysin gene (*hld* gene) expression was detected using quantitative real-time PCR (qRT-PCR). This study used the 16srRNA Housekeeping gene as a calibration. Prepared the primers was according to the supplied company. Measured gene expression in the hVISA isolate before and after treatment with *P. harmala* seeds essential oil. The doses of the *P.harmala* seeds essential oil employed for the test were below the MIC value to enable bacterial growth with resistance induction.

**Extraction RNA from isolates**

Total RNA extract obtained by TRIzol Reagent method described by (5). prepared Mueller–Hinton broth (Oxoid, UK) tubes with the appropriate concentration of *P. harmala* seeds essential oil (0.39 mg/ml); Two ml after treated hVISA with *P. harmala* seeds extract sample was taken from dose under the MIC value and before treated (control) and collected in a tube containing TRIzolTM Reagent for RT-qPCR test. All samples in this study had total RNA extracted successfully. RNA concentrations varied from 70 to 160 ng/µl from the control group. At the same time, from the treated group, it varies from 80 to 160 ng/µl. The control group had purity ranging from 1.7 to 1.9. While RNA treated samples' purity ranged from 1.8 to 1.9. Primer

reactions, the housekeeping gene and the *hld* gene were required. Later, determined the efficiency of cDNA concentration using qPCR efficiency, with all steps resulting in a perfect yield, indicating that reverse transcription was effective.

**Quantitative real-time polymerase chain reaction (qRT–PCR) :** This study used Mic real-time PCR cycler (BioMolecular System, Australia) for quantitative real-time PCR (qRT–PCR). the master amplification reaction used to amplify a segment of mRNA using the One-Step RT-PCR program. To test the expression of the (*hld*) gene, we used the kit GoTaq®1-Step RT-qPCR System (Promega, USA) reagent system with a one-step RT-qPCR procedure to do quantitative RNA analysis. The method used a 10 µl reaction volume per the manufacturer's instructions. The total RNA volume required for reverse transcription was (1µl).The expression levels of (*hld*) genes were measured using the Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) method, a sensitive method for evaluating steady-state mRNA levels. This study confirmed the target gene's expression using the quantitative real-time qRT-PCR SYBR Green assay. The *hld* gene primer sequences, forward primers (FP) 5'-ATTTGTTCACTGTGTCGATAATCC-3' and reverse primer (RP) 5'-GGAGTGATTTCAATGGCACAAG-3', were used for the *hld* gene (42). FP 5'-CTGCTGCCTCCCGTAG-3' and RP 5'-CCGACCTGAGAGGGTGA-3' for 16srRNA (19). Macrogen (Korea) synthesized the primers and kept it lyophilized at (-20°C). The expression of a few genes was normalized against housekeeping genes. For data analysis, the ΔΔCt method used for data analysis.

## RESULTS AND DISCUSSION

**Chemical compound of *Peganum harmala* L. essential oils:** In this study was extracted the *P. harmala* seeds' essential oils by the ethanolic extract process; the seeds' yields (v/w) % based on the fresh weight represented 12%. Then analyzed the chemical compositions of *P. harmala* seeds extracted by GC-MS (Table1); in comparison to the constituents of the NIST11 library, 22 peaks identified.Twenty two compounds in the extract accounted for 99.62% of the seed extract. Table 1 shows the types and amounts of essential oil compounds and the retention time indices for the identified mixture. The outcomes showed that the highest compounds in seeds was Harmine (58.26%), followed by 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (28.55%), observed Hexadecanoic acid, methyl ester (4.30%), then Linoleic acid ethyl ester (3.32%), and Methyl stearate (2.12%). Sassoui *et al*. (40) pointed out the components of *P. harmala* essential oil, which is grown in Algeria, contained: (48%) harmaline, (38%) harmine, (8%) tetrahydroharmine, (0.06%) tetrahydroharman, and (0.05%) 6-methoxytetrahydro- 1-norhar- manone. Asadzadeh *et al*. (5) indicated the main composition of *P. harmala* seeds extracts grown in Iran was (28.32%) 2,3-dimethyl benzofuran and (7.46%) cis-linalool oxide. The different primary components of the extracted essential oil are due to the various other cultured areas of the plant. Where the change in the geographical nature and environmental conditions of the region has a significant impact on the quality and quantity extracted from the same species and cultured in different areas (5). the methods used to extract the plants affect the quality and quantity of the produced essential oil (12,22).

**Identification of *Staphylococcus aureus***

With a traditional culture technique and microscopic feature, 89 (53.9%) of 165 inpatient and outpatient samples were identify as *staphylococcus aureus*. Eighty-nine isolates produced smooth, translucent, creamy, yellow-pigmented colonies on mannitol salt agar and fermented mannitol. It had gram stain reactivity and looked like grape clusters, but it wasn't spore-forming or motile. In biochemical assays, 89 isolates tested positive for coagulase and catalase but negative for oxidase.

**Detection of methicillin-resistant *Staphylococcus aureus:*** Methicillin-resistant *Staphylococcus aureus* isolated by cultivating the isolates on CHROMagar$^{TM}$ MRSA medium and utilizing the cefoxitin disk method and traditional PCR. CHROMagar$^{TM}$ MRSA medium inhibits all MSSA isolates while allowing all MRSA

**Table 1. Chemical composition of essential oil of *Peganum harmala L.* seeds extract**

| Reaction Time | Constituent | Area % |
|---|---|---|
| **3.672** | **Pyridine** | **0.08** |
| **12.033** | **2-Vinyl-9-[.beta.-d-ribofuranosyl] hypoxanthine** | **0.07** |
| **19.034** | **cis-Vaccenic acid** | **0.06** |
| **19.983** | **6-Octadecenoic acid, (Z)-** | **0.35** |
| **20.222** | **Hexadecanoic acid, methyl ester** | **4.30** |
| **20.624** | **Clonitazene** | **0.12** |
| **21.043** | **Hexadecanoic acid, ethyl ester** | **0.78** |
| **21.462** | **Trimethyl(cyclohexylmethoxy)silane** | **0.20** |
| **22.343** | **9,12-Octadecadienoic acid (Z,Z)-, methyl ester** | **28.55** |
| **22.616** | **Methyl stearate** | **2.12** |
| **23.043** | **Linoleic acid ethyl ester** | **3.32** |
| **23.343** | **1H-Cyclopenta(b)quinoline, 2,3,5,6,7,8-hexahydro-9-amino-** | **0.19** |
| **24.172** | **Oleic Acid** | **0.12** |
| **24.565** | **2-Methyl-Z,Z-3,13-octadecadienol** | **0.13** |
| **24.813** | **Methyl 5,9-dimethyldecanoate** | **0.22** |
| **26.198** | **5-Phenyl-1,3-diazaadamantane** | **0.10** |
| **26.523** | **Harmine** | **58.26** |
| **28.079** | **Harmine, ethenyl(ester)** | **0.15** |
| **28.250** | **Harmine, ethenyl(ester)** | **0.06** |
| **28.309** | **Benzoic acid, 2,4,5-trimethoxy-** | **0.08** |
| **28.728** | **3,4-Dimethylbenzo[4,5]imidazo[1,2- a]pyridin-1-ol** | **0.10** |
| **29.575** | **4,4'-Diisopropylbiphenyl** | **0.26** |

Bold values represent the majority compounds of the essential oil

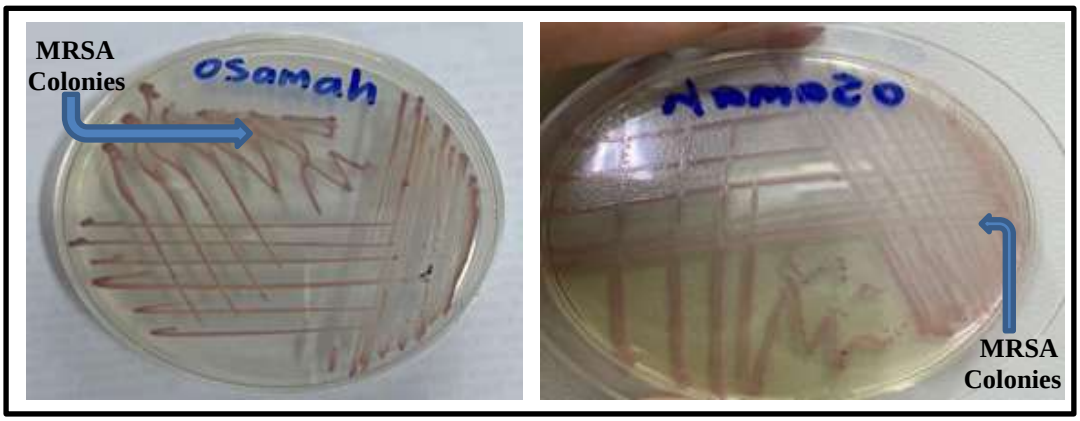


**Figure 1 . MRSA isolate on CHROMagar™ MRSA media**

isolates to grow; 30 of the 89 isolates were rose to mauve color as MRSA (Figure 1). Chromogenic agar facilitated the isolation and identification of MRSA from direct isolation plates for 24 hrs., reducing the requirement for additional biochemical assays (25); the approach is cost-effective, saves time and produces strong results similar to the PCR method (50). However, cefoxitin disc resistance is the marker for MRSA screening (10). For detecting MRSA, PCR-based techniques are the most reliable to confirm *mecA* gene (37). Among 89 *S.aureus* isolates, 30 (33.7 %) were MRSA, with 19 (63.3 %) and 11 (36.7 %) from inpatients and outpatients, respectively. This study almost agreed with an Iraqi survey done by Shaima'a (41), who found that 44% of MRSA isolates were hospital-acquired while 34% were community acquired, MRSA was identified using the Latex agglutination method, which detects PBP2a in *S. aureus* isolates. And PCR to recognize the *mecA* gene (41)/

**Identification of Hvisa**

The PAP-AUC method determined that one of the thirty MRSA isolates (3.3%) showed the hVISA phenotype. This hVISA isolated from the patient who had hVISA bacteremia, was an old man (age 73 years).PAP-AUC is the gold standard for identifying hVISA; this is too time-intensive to be employed on a routine basis, promoting the development of other screening methods such as BHIV4 and Etest (16). PAP-AUC is the gold standard for

identifying hVISA, and this study depending of this method for identify hVISA. This study found two (6.6 %) hVISA in MRSA isolates; according to Etest, the MIC values were ≥8 µg/ml in positive isolates. Chaudhari et al. (9) observed that the prevalence of hVISA was 6.9% with PAP‑AUC and macro method Etest. Walsh et al. (48) developed the Etest macro method with 8 µg/ml vancomycin breakpoints, which was specific and sensitive for verification of hVISA but is too costly for routine screening. Before confirming positive screening tests by the PAP method, the isolated screened by BHIV4 and Etest; most of the studies initially used a variety of screening tests. In a study in Turkey, Sancak *et al*. (39) tested 256 clinical MRSA isolates from 256 individuals. They confirmed positive results using the PAP-AUC technique after screening with BHI agar containing vancomycin; their research discovered 46 (17.97%) hetero VISA isolates. The rate of hVISA was 2 (6.6%) by BHIV4 and Etest but 1 (3.3%) by the PAP-AUC method. This study confirmed 1 (3.3%) as hVISA, with 3 strains (10%) as VISA and one strain as VSSA by the PAP-AUC method, which remains one of the gold standard techniques for confirming VISA\hVISA from specimens (Figure 2). Prevalence rate of hVISA is consistent with a study by Iyer and Hittinahalli from India that shows an hVISA prevalence rate of 2% (17). The case of Japan showed hVISA prevalence to be 3% (34/1,149 MRSAs) (14); in Italy, Germany, France, and The Netherlands, 1.1%, 0.21%, 0.6%, and 6%, respectively (7,27,34,46). Thus, hVISA prevalence appears to occur with slight differences among countries, although this is partially due to detection methods. This study results indicate that hVISA isolates seem to be more frequent in blood-borne; this is consistent with Zhang *et al*. (55), who found hVISA frequencies in blood culture samples. Moore *et al.* (26) confirmed vancomycin treatment failure in MRSA endocarditis caused by an hVISA strain. Maor *et al*. (29) compared 27 patients with hVISA bacteremia to 223 healthy volunteers as controls (MRSA bacteremias). They observed that hVISA bacteremia links to more prolonged infection and a greater risk of endocarditis and osteomyelitis compared to controls. *In vitro* pharmacokinetic /pharmacodynamic study, Rose *et al*. (35) showed that vancomycin had limited efficacy against clinical strains of hVISA at suitable dosages (AUC/MIC, 105–317).

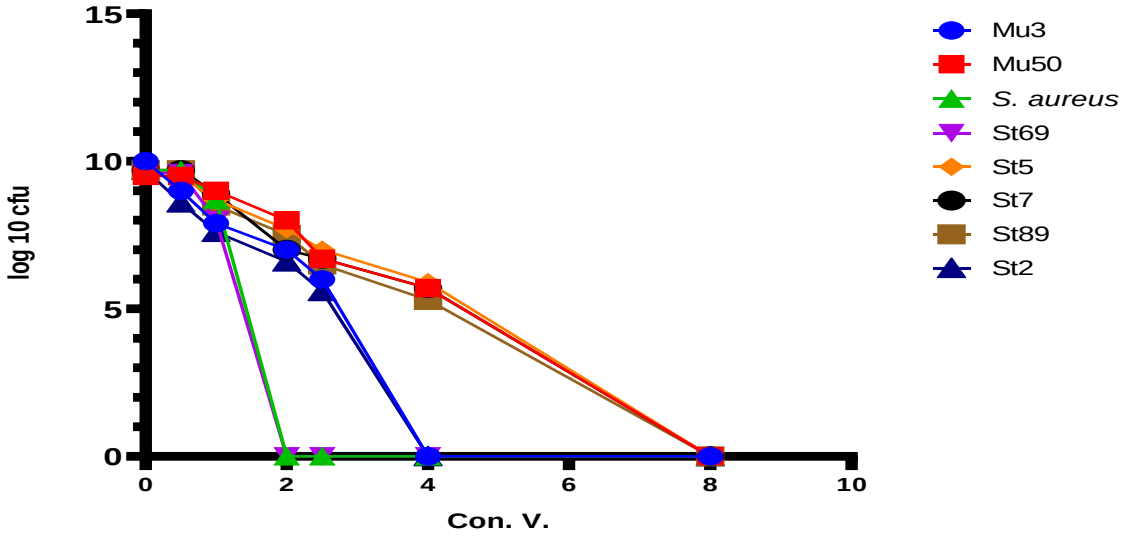


**Figure 2. Compares the population analysis profiles of ATCC 25923 (VSSA), ATCC700698 (Mu3-hVISA), and ATCC700699 (Mu50-VISA) control strains with representative test strains (St69, St2, and St5, St7, St89). The total number of colonies grown on (BHI) agar showed with varied vancomycin doses and incubation was 48 h. the positive control strains Mu50, and Mu3, with test strains St2, St5, St7, and St89, show reduced vancomycin susceptibility (≥4 µg/mL). The negative control strain ATCC 25923 shows increased vancomycin susceptibility (<4 µg/mL).**

**Estimation of the ethanolic extract's minimum inhibitory concentration:** This study evaluated the antibacterial activity of *P. harmala* seeds extracts using MIC assays. *P. harmala* essential oil has antibacterial activity on the tested strain; heterogeneous vancomycin intermediated *Staphylococcus aureus* (hVISA). The results showed that the MIC for hVISA isolate was 3.9 mg/ml, which was enough to stop the growth of hVISA, which was the most susceptible to these results.The result is lower than Benbott *et al*. (6), who discovered a lower inhibitory concentration of 100 (mg/ml) for *Staphylococcus aureus, Staphylococcus saprophyticus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis* and *Serratia* spp., Darabpour *et al.* (11) found that *P. harmala* seeds essential oil inhibited *S. aureus, Streptococcus pyogenes, Streptococcus epidermidis, Bacillus cereus, Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeroginosa* and *Escherichia coli* At 50-400 mg/mL doses, Ali *et al*. (4) also investigated the antibacterial effects of *P. harmala* essential oil. It was effective against all strains listed below (*Acinetobacter calcoaceticus, S. aureus* and *Candida albicans*). The different antibacterial levels of various *P. harmala* extracts could be due to various solubility of different combinations observed in *P. harmala,* specifically when used solvents with specialized antifungal or antibacterial actions. The seeds and roots extracts from *P. harmala* have been shown in numerous studies to have a more significant antibacterial effect than extracts from other plant parts (28). Antibacterial effects of *P. harmala* seeds due to the chemical composition, especially the presence of alkaloid (harmine) linoleic acid (9, 12-Octadecadienoic acid (Z,Z)-, methyl ester), and they are well known for antimicrobial activity (1,8,17).

**Gene expression analysis**

RT-qPCR has become the gold standard for gene expression analysis because it is very accurate, sensitive, and efficient. It is different from other gene expression techniques; it is crucial to realize that in a relative quantification investigation, the experiments are usually interested in comparing the expression level of a particular gene across multiple samples. RT-PCR-based real-time is a preferred measurement method because it is one of the most sensitive detection methods that provide the actual number and repeatable, multi-analysis in many samples in a short time (23). The current experiment employs the SYBR green, a fluorescent dye that identifies any double-stranded DNA, including cDNA. In this study, quantitative RT-PCR measured the mRNA expression (*hld* gene) responsible for the production of delta-hemolysin with the Housekeeping gene (H.K) as a calibration by comparing the hVISA growth after and before treatment with *P. harmala* seeds extract. The amplification was recorded using the Ct values of genes from the quantitative RT PCR program; High Ct values show low gene expression, while low Ct values show increased gene expression. The results showed a decrease in gene expression after being treated with *P. harmala* essential oil, as shown in table 2. One of the virulence factors that *agr* controls are Delta-hemolysin, which differs from the other factors that *agr* controls. The *hld* gene inside the *agr* locus encodes this protein, produced from the translation of RNAIII, the *agr* effector molecule (52); as the result, this study employed the expression *hld* gene as a functional marker *agr* in an hVISA strain. The gene expression level was normalized to that of a housekeeping gene and measured using the Ct value and folding (2 Ct) methods. Table 2 and figure 3 shows the expression *hld* gene according to treatment with *p. harmala* seed extract. The mean Ct value of *hld* gene amplification was (18.92) in control. The Ct values treatment was a mean (20.60). A significant difference in the mean increased Ct values indicates low gene expression. Expression of the *hld* gene was low after treatment. The results of this study show that *hld* gene expression is a useful tool for identifying hVISA resistance programs. The expression of the *hld* gene was reduced after treatment with *p. harmala* seed oile at a concentration of 0.3 mg/ml, indicating that more research is needed to understand the effects of other plant extracts on the *hld* gene expression.

**Table 2. Genetic expression values for *hld* gene after and before treatment**

| Sample | 16srRNA | *Hld* | DCT | DDCT | Folding |
|---|---|---|---|---|---|
| hVISA | 9.10 | 18.92 | 9.82 | 0.00 | 1.0 |
| hVISA Treated | 9.85 | 20.60 | 10.75 | 0.93 | 0.5 |

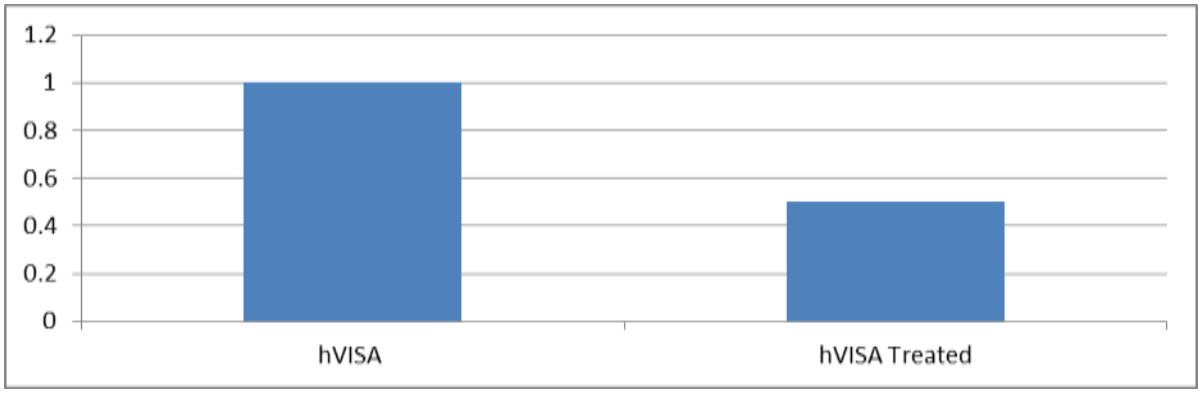


**Figure 3. Inhibitory effect of the *P. harmala* essential oil on the hVISA strain, relative expression ratios (ratio of gene to action) of (*hld* gene) gene encoding delta hemolysin. And housekeeping gene 16srRNA as a normalize transcription of *hld* by the comparative CT method**

## REFERENCES

1.Abdel Karim, M., A. Sufian, M. S. Kamal, and O. Inas, 2017. GC–MS analysis and antimicrobial activity of fixed oil from Saudi *Lepidium sativum* (crusifereae) seeds. Int. J. Adv. Res. 5(3): 1662-1670

2.Abou-Elkhair, E., H. Fadda, and U. Abu-Mohsen, 2010. Antibacterial activity and phytochemical analysis of some medicinal plants from Gaza strip-Palestine. Journal of Al-Azhar University-Gaza. 12: 45-54.

3.Ahmad, H., N., Ali, B. Ahmad and I. Khan, 2012. Screening of *Solanum surrattense* for antibacterial, antifungal, phytotoxic and haemagglutination. Journal of Traditional Chinese Medicine. 2012; 32(4): 616-620.

4.Ali, N.H., S. Faizi, and S. U. Kazmi, 2011. Antibacterial activity in spices and local medicinal plants against clinical isolates of Karachi. Pakistan. Pharmaceutical Biology. 49(8): 833-839

5.Asadzadeh, R., N. Abbasi, and M. Bahmani,2021. Extraction and identification of chemical compounds of *Peganum harmala* L. seed essential oil by HS-SPME and GC-MS methods. Traditional and Integrative Medicine. 6 (3):235-229

6.Benbott, A., A. Yahyia, and A. Belaidi, 2012. Assessment of the antibacterial activity of crude alkaloids extracted from seeds and roots of the plant *Peganum harmala* L. Journal of Natural Product and Plant Resources. 2(5): 568-573.

7.Bierbaum, G., K. Fuchs, W. Lenz, C. Szekat and H. G. Sahl, 1999. Presence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Germany. European. Journal of Clinical Microbiology and Infectious Diseases. 18(10): 691-696.

8.Breine, A., M. Van Gysel, M. Elsocht, C. Whiteway, C. Philippe, T. Quinet, A. Valcek, J. Wouters, S. Ballet and C. Van der Henst, 2021. Antimicrobial activity of a repurposed harmine-derived compound on extensively drug-resistant *Acinetobacter baumannii* clinical isolates. Front. Cell. Infect. Microbiol. 11:789672

9.Chaudhari, C. N., K. Tandel, N. Grover, S. Sen, P. BhattA. K. Sahni and A. K. Praharaj, 2015.Heterogeneous vancomycin-intermediate among methicillin resistant *Staphylococcus aureus*. Medical Journal Armed Forces India. 71(1):15-18.

10.Clinical and Laboratory Standards Institute (CLSI). 2021. Performance Standards for Antimicrobial Susceptibility Testing, Thirty one Informational Supplement. CLSI Document M100-Ed 31, Clinical and Laboratory Standards Institute, Wayne, PA, USA, PP: 1-352

11.Darabpour, E., A. P. Bavi, H. Motamedi, and S. M. S. Nejad, 2011. Antibacterial activity of different parts of *Peganum harmala* L. growing in Iran against multi-drug resistant bacteria. EXCLI Journal. 10: 252-263

12.Farjadmand, F., M. Khanavi, M. Eftekhari, A. Hosseinsalari, T. Akbarzadeh, M. Safavi, R. Asatouri, M. Mirabzadeh, and M. R. Shams Ardekani, 2018. The effect of extraction method on the major constituents and biological effects of *Trachyspermum ammi* L. fruits. Research Journal of Pharmacognosy. 5(1), 55-61.

13. Hassan, T. J., and S. I., Hussein, 2022. Development of bioprocesses for production and purification of l-asparaginase from *Staphyllococcus aureus*, and *in vitro* efficacy against human breast cancer cell line. Iraqi Journal of Agricultural Sciences,53(6):1525-1538. https://doi.org/10.36103/ijas.v53i6.1668

14.Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi and I. Kobayashi, 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. The Lancet. 350(9092): 1670-1673

15.Holmes, N. E., P. D. Johnson, and B. P. Howden, 2012. Relationship between vancomycin-resistant *Staphylococcus aureus*, vancomycin-intermediate *S. aureus*, high vancomycin MIC, and outcome in serious *S. aureus* infections. Journal of Clinical Microbiology. 50(8): 2548-2552.

16. Howden, B.P., J. K. Davies, P. D. Johnson, T. P. Stinear, and M. L. Grayson, 2010. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clinical Microbiology Reviews. 23(1): 99-139.

17.Iyer, R.N. and V. Hittinahalli, 2008. Modified PAP method to detect heteroresistance to vancomycin among methicillin resistant *Staphylococcus aureus* isolates at a tertiary care hospital. Indian Journal of Medical Microbiology. 26(2): 176-179.

18.Jaddoa, N. T. and H. J. F Al-Mathkhury, 2018. Biofilm shows independency form hemolysin genes arsenal in methicillin-resistant *Staphylococcus aureus*. Iraqi Journal of Science. 59(4C): 2184-2194

19. Jomehpour, N., G. Eslami, and M. B. Khalili, 2016. The effect of *Ferula assa-foetida* L and *Carum copticum* hydroalcoholic extract on the expression levels of *Staphylococcus aureus* genes involved in quorum sensing. Jundishapur Journal of Microbiology. 9(10): e33879.

20.Kőszegi, K., G. Vatai, and E. Békássy-Molnár, 2015. Comparison the soxhlet and supercritical fluid extraction of nettle root (*Urtica dioica* L.). Periodica Polytechnica Chemical Engineering. 59(3): 168-173.

21.Lee, M.Y., W. I. Lee, M. H. Kim, S. Y. Kang, and Y. J. Kim, 2020. Etest methods for screening heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) strains. Current Microbiology. 77(10): 3158-3167.

22.Leite, B. 2012. Extraction of Essential Oils from Vetiver (Vetiveria Zizanioides) Grass. Ph.D. Dissertation. University of Kwa Zulu Natal. South Africa. PP: 30-100.

23.Lo, S. F., T. D. Ho, Y. Liu, M. Jiang, K. Hsieh, K. Chen, L. Yu, M. Lee, C. Chen, T. Huang, M. Kojima, H. Sakakibara, L. Chen and S. Yu, 2017. Ectopic expression of specific GA2 oxidase mutants promotes yield and stress tolerance in rice. Plant Biotechnol. J.15(7):850–864.

24.Mahmoudian, M., H. Jalilpour and P. Salehian, 2002. Toxicity of *Peganum harmala*: Review and a case report. Iranian Journal of Pharmacology and Therapeutics. 1(1): 1-4.

25.Malhotra-Kumar, S., K. Haccuria, M. Michiels, M. Ieven, C. Poyart, W. Hryniewicz, and H. Goossens, 2008. Current trends in rapid diagnostics for methicillin resistance *Staphylococcus aureus* and glycopeptides resistant Enterococcus species. J. Clin. Microbiol. 46(5):1577-1587

26.Maor, Y., M. Hagin, N. Belausov, N. Keller, D. Ben-David and G. Rahav, G. 2009. Clinical features of heteroresistant vancomycin-intermediate *Staphylococcus aureus bacteremia* versus those of methicillin-resistant *S. aureus* bacteremia. Journal of Infectious Diseases. 199(5): 619-624.

27.Marchese, A., G. Balistreri, E. Tonoli, E. A. Debbia, and G. C. Schito, 2000. Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. Journal of Clinical Microbiology. 38(2): 866-869

28.Mohsenipour, Z. and M. Hassanshahian, 2016. Antibacterial activity of Espand (*Peganum harmala*) alcoholic extracts against six pathogenic bacteria in planktonic and biofilm forms. Biological Journal of Microorganism. 4(16):109–120

29.Moore, M. R., F. Perdreau-Remington, and H. F. Chambers, 2003. Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate *Staphylococcus aureus* in a patient with endocarditis and in the rabbit model of endocarditis. Antimicrobial Agents and Chemotherapy. 47(4): 1262-1266.

30. Muntaha M. Hadi and Majid Rasheed Majeed .2023. Production of green dettol as bacterial inhibitor and disinfectant, Iraqi Journal of Agricultural Sciences, 54(4), 1008–1015. https://doi.org/10.36103/ijas.v54i4.1789

31.Novick, R. P. 2000. Pathogenicity Factors and Their Regulation. In Fischetti, V. A., R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood, (ed.) Gram-positive pathogens. ASM Press, Washington, D.C. pp. 392–407

32.Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth and S. Moghazeh, 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. The EMBO Journal. 12(10): 3967-3975.

33.Patel, J. D. and V. Kumar, 2008. *Annona squamosal* L.: phytochemical analysis and antimicrobial screening. Journal of Pharmacy Research. 1(1): 34-38

34.Reverdy, M., S. Jarraud, S. Bohin-Duhreux, E. Buret, P. Girardo, G. Lina, F. Vandenesch, and J. Etienne, 2001. Incidence of *Staphylococcus aureus* with reduced susceptibility to glycopeptides in two French hospitals. Clinical Microbiology and Infection. 7(5): 267-272.

35.Rose, W. E., S. N. Leonard, K. L. Rossi, G. W. Kaatz, and M. J. Rybak, 2009. Impact of inoculum size and heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) on vancomycin activity and emergence of VISA in an *in vitro* pharmacodynamic model. Antimicrobial Agents and Chemotherapy. 53(2): 805-807.

36.Saeidi, S., A.N. Boroujeni, H. Ahmadi and M. Hassanshahian, 2015. Antibacterial activity of some plant extracts against extended-spectrum beta-lactamase producing escherichia coli Isolates. Jundishapur J. Microbiol. 8(2): e15434.

37.Sajith Khan A. K., P. J. Shetty, L. Y. Sarayu. A. Chidambaram and R. Ranganathan, 2012. Detection of *mecA* gene of methicillin-resistant *Staphylococcus aureus* by polymerase chain reaction. International Journal of Health and Rehabilitation Sciences. 1(2): 64-68.

38.Sakoulas, G., G. M. Eliopoulos, V. G. Fowler Jr, R C. Moellering Jr, R. P. Novick, N. Lucindo, M. R. Yeaman and A.S. Bayer, 2005. Reduced susceptibility of *Staphylococcus aureus* to vancomycin and platelet microbicidal protein correlates with defective autolysis and loss of accessory gene regulator (*agr*) function. Antimicrobial Agents and Chemotherapy. 49(7), 2687-2692.

39.Sancak, B., S. Ercis, D. Menemenlioğlu, S. Çolakoğlu, and G. Hasçelik, 2005. Methicillin-resistant *Staphylococcus aureus* heterogeneously resistant to vancomycin in a Turkish University Hospital. Journal of Antimicrobial Chemotherapy. 56(3): 519-523.

40.Sassoui, D., R. Seridi, K. Azin, and M. Usai, 2015. Evaluation of phytochemical constituents by GC-MS and antidepressant activity of *Peganum harmala* L. seeds extract. Asian Pacific Journal of Tropical Disease. 5(12): 971-974.

41.Shaima'a R. H. 2011. Identification of Healthcare-associated and Community-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates. M. Sc. Thesis. College of Medicine/ Baghdad University. Iraq. pp: 54-92.

42.Sharifi, A., A. Mohammadzadeh, T. Zahraei Salehi, and P. Mahmoodi, 2018. Antibacterial, antibiofilm and antiquorum sensing effects of *Thymus daenensis* and *Satureja hortensis* essential oils against *Staphylococcus aureus* isolates. Journal of Applied Microbiology. 124(2): 379-388.

43.Singh, A., K. N. Prasad, R. Misra, M. Rahman, S. K. Singh, R. P. Rai, A. Tripathi and J. K. Srivastava, 2015. Increasing trend of heterogeneous vancomycin intermediate *Staphylococcus aureus* in a tertiary care center of northern India. Microbial Drug Resistance. 21(5): 545-550.

44. Tehrani, S.S.H., S.H.S. Shabani, S.T. Enferadi and Z. Rabiei, 2014. Growth inhibitory impact of *peganum harmala* L. On

two breast cancer cell lines. Iran J Biotech. 12(1):8–14.

45.Tong, S.Y.C., J.S. Davis, E. Eichenberger, T.L. Holland and V. G. Fowler Jr. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews. 28(3): 603-661.

46.Van Griethuysen, A., A. Van't Veen, A. Buiting, T. Walsh and J. Kluytmans, 2003. High percentage of methicillin-resistant *Staphylococcus aureus* isolates with reduced susceptibility to glycopeptides in the Netherlands. Journal of Clinical Microbiology. 41(6): 2487-2491.

47. Ventola, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and Therapeutics. 40(4): 277-283

48.Walsh, T.R., A. Bolmström, A. Qwärnström, P. Ho, M. Wootton, R. A. Howe, A. P. Macgowan and D. Diekema, 2001. Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. Journal of Clinical Microbiology. 39(7): 2439-2444.

49.William, B. W., D. V. paul, M. G. George, J. Dorothy, R. K Noel, L. Wolfgang, A. R. Fred and S. Karl, 2009. Bergey's Manual of Systematic. 2nd edition. Com. pp: 392-433

50.Wolk, D. M., J. L. Marx, L. Dominguez, D. Driscoll and R. B. Schifman, 2009. Comparison of MRSA select agar, CHRO Magar methicillin- resistant *Staphylococcus aureus* (MRSA) medium, and Xpert MRSA PCR for detection of MRSA in nares: diagnostic accuracy for surveillance samples with various bacterial densities. Journal of Clinical Microbiology. 47(12): 3933-3936.

51.Wootton, M., R. A. Howe, R. Hillman, T. R. Walsh, P. M. Bennett and A. P. MacGowan, 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. Journal of Antimicrobial Chemotherapy. 47(4): 399-403

52.Wright, J. S., G. L. Lyon, E. A. George, T. W. Muir, and R. P. Novick, 2004. Hydrophobic interactions drive ligand-receptor recognition for activation and inhibition of staphylococcal quorum sensing. Proceedings of the National Academy of Sciences. 101(46): 16168-16173.

53.Yassin, H.Y., A.K. Melconian and S.S. Mahmood, 2022. Prevalence of exfoliative toxin genes among clinical isolates of *staphylococcus aureus* in Iraq. Iraqi Journal of Agricultural Sciences. 53(2), 465-470. https://doi.org/10.36103/ijas.v53i2.1554

54. Zha, X., P. Zhao, F. Gao and Y. Zhou, 2020. Complete chloroplast genome sequence of *Peganum harmala*, an important medicinal plant. Mitochondrial DNA Part B. 5(1): 652-653.

55.Zhang, S., X. Sun, W. Chang, Y. Dai and X. Ma, 2015. Systematic review and meta-analysis of the epidemiology of vancomycin-intermediate and heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates. PloS One. 10(8): e0136082.