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Gene Expression of Aminoglycosides Resistant Genes in *Staphylococcus aureus* Isolated from Wound and Burn Infection by Some Phytochemical Compounds

Thesis

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التعبير الجيني لجينات مقاومة الامينوكلايكوسيد في بكتيريا المكورات العنقودية الذهبية المعزولة من اصابات الجروح والحروق بإستخدام بعض المركبات الكيميائية النباتية

اطروحة مقدمة إلى
مجلس كلية العلوم – جامعة بابل
وهي جزء من متطلبات نيل شهادة الدكتوراه/ فلسفة في العلوم /علوم الحياة

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Summary

Burn and wound destroys the physical skin which the natural barrier to the external environment. As a result the burned area is prone to infection and colonization of microorganisms. The most common pathogenic colonizing bacteria are *Staphylococcus aureus* . *S. aureus* is both a human commensal bacterium, as it is carried by a significant amount of individuals, and a possible infectious pathogen.

The current study included the collection of 230 samples from burns and wounds patients from Imam Al-Sadiq Hospital, Al-Hilla teaching Hospital and Specialized Burn Center in Medical City of Baghdad and Kufa from February to September 2022. The swabs had been cultured on different media; the colonies were diagnosed based on the phenotypic and culture characteristics. The bacteria were identified through cultural characters, Gram staining and diagnosed by VITEK® 2 Compact Automated Systems. It was found that out of 230 samples only 63 isolates were *Staphylococcus. aureus* .

The Tissue Culture Plate (TCP) method showed that 55 *Staphylococcus aureus* isolates isolated from wounds and burns had a high degree of biofilm-forming ability and that 8 of these isolates were not biofilm-producing.

In Modified Kirby-Bauer method to test the sensitivity of bacteria towards Aminoglycosides antibiotics, which included Amikacin (10 µg), Gentamicin (10 µg), Streptomycin (25 µg), Neomycin (30 µg), Tobramycin (10 µg), and Kanamycin (30 µg). The results were gave the highest resistance against the antibiotic Kanamycin, and the lowest

resistance against Amikacin and Gentamicin, with a ratio 63, 4%, 47, 6% respectively.

Some *S. aureus* have the ability to produce aminoglycoside modifying enzymes (AMEs) as the main mechanism of resistance to this antibiotic. Therefore, during this study ,it was investigated the prevalence of AME genes which are *aac (6 ') Ie / aph (2 "), aph (3') - IIIa1, ant (4 ') - Ia1* besides *mecA* gene for *S. aureus* strains isolated from burn wound specimens, 12 isolates (24%) contained *mecA* gene. In addition, 32 isolates (64 %) had *aph(3')-IIIa1* gene and 6 isolates (12 %) had *aac(6')Ie/aph(2")*, While the presence of the *ant(4')-Ia1* gene was not observed in any of the samples.

In order to solve the problem of bacterial resistance to antibiotics, some medicinal plants were used to determine their effectiveness against bacteria. Among these plant extract curcumin from plants *Curcuma longa* and berberine from plant *Berberis aristata*. The result thus obtained suggests that bioactive principles of these plants can be used particularly against these bacteria of clinical origin, Where curcumin proved its inhibitory efficacy at concentration 200 mg/ml in (25.25 ± 4.089) , while berberine at concentration 250 mg/ml had inhibitory efficacy with (31.60 ± 2.501) with (*P value* = 0.05) .

After confirming the effectiveness of plant extracts as an anti- *S. aureus*, its effect on cells was studied by studying cytotoxicity activity in tissue cultures by using MTT. For curcumin on human breast cancer (MCF-7) cells *in vitro* compared to human hepatic (WRL-68) cells assay, and the effect of berberine on skin cancer cells (A431) and normal human keratinocyte cells (HaCaT). The results showed high cytotoxicity of curcumin against MCF-7 cells at the concentration (400 µg/ml). The cell

viability was (37.62± 3.451) and the IC_{50} value was (269), while the bioactivity was very low towards WRL-68 cells at the concentration (400 µg/ml), it was (77.74±3.210) and the IC_{50} value is (919).

As for the berberine, its cytotoxicity against A431 cells was high at the concentration (400 µg/ml), with cell viability (47.96± 3.966) and an IC_{50} value (363), while its cytotoxicity was low at the same concentration towards HaCaT cells (74.58± 6.078) and a high IC_{50} value. (807) Thus, we conclude that curcumin and berberine are a valuable source of anti-cancer drugs with safe and selective effectiveness on cancer cells compared to normal human cells.

For studying the gene expresstion of are *aac (6 ') Ie / aph (2 "), aph (3') - IIIa1,* and *mecA* gene for *S. aureus* strains isolated from burn wound specimens, a total of RNA was isolated from *S. aureus* using the TRIzol purification kit and reversed transcripted to cDNA before being submitted for further amplification to investigate the gene expression of these genes using the RT-qPCR methodology before and after treatment with curcumin and berberine. The findings described the gene expression of are *aac (6 ') Ie / aph (2 "), aph (3') - IIIa1,* and *mecA* gene in connection to housekeeping genes; the genes demonstrated changes and decreasing the level of gene expression , for *aac (6 ') Ie / aph (2 ") , aph (3') - IIIa1,* and *mecA* gene was (0.69) and (0.91) , (0.55) and (0.92) , (0.24) and (0.69) after being treated with curcumin and berberine respectively, . This result implies that the bacteria's growth rate and gene expression of *aac (6 ') Ie / aph (2 "), aph (3') - IIIa1,* and *mecA* gene decreased after treatment with curcumin and berberine.

الخــلاصـــة

الحروق والجروح تدمر الجلد الذي يشكل الحاجز الطبيعي للبيئة الخارجية. ونتيجة لذلك، تصبح المنطقة المحروقة عرضة للعدوى واستعمار من قبل الكائنات الحية الدقيقة. البكتيريا المسببة للأمراض الأكثر شيوعًا هي المكورات العنقودية الذهبية. حيث تعتبر من بكتيريا مترافقة مع الإنسان، كما أنها من مسببات الأمراض المعدية المحتملة.

تضمنت الدراسة الحالية جمع ٢٣٠ عينة من مرضى الحروق والجروح من مستشفى الامام الصادق ومستشفى الحلة التعليمي ومركز الحروق التخصصي في مدينة الطب ببغداد والكوفة للفترة من شباط الى ايلول ٢٠٢٢. وقد تم زراعة المسحات على اوساط زرعية مختلفة ؛ تم تشخيص المستعمرات على أساس الخصائص المظهرية والزرعية. تم التعرف على البكتيريا من خلال الخصائص الزرعية وصبغة غرام وتشخيصها بواسطة الأنظمة الآلية المدمجة VITEK® ٢. وقد وجد أنه من بين ٢٣٠ عينة كانت هناك ٦٣ عزلة فقط من المكورات العنقودية الذهبية .

أظهرت طريقة زراعة الأنسجة (TCP) أن ٥٥ عزلة من المكورات العنقودية الذهبية المعزولة من الجروح والحروق لديها درجة عالية من القدرة على تكوين الأغشية الحيوية وأن ٨ من هذه العزلات لم تكن منتجة للأغشية الحيوية.

بطريقة كيربي باور المعدلة لاختبار حساسية البكتيريا تجاه المضادات الحيوية الأمينوكلايكوسيديه والتي تشمل أميكاسين (١٠ ميكروغرام)، جنتاميسين (١٠ ميكروغرام)، ستربتومايسين (٢٥ ميكروغرام)، نيومايسين (٣٠ ميكروغرام)، توبراميسين (١٠ ميكروغرام)، وكاناميسين (٣٠ ميكروغرام). أظهرت النتائج أعلى مقاومة ضد المضاد الحيوي كاناميسين، وأقل مقاومة ضد أميكاسين وجنتاميسين بنسبة ٦٣، ٤%، ٤٧، ٦% على التوالي.

تتمتع بعض المكورات العنقودية الذهبية بالقدرة على إنتاج إنزيمات تعديل الأمينوغليكوزيد (AMEs) باعتبارها الآلية الرئيسية لمقاومة هذه المجموعة من المضادات الحيوية . لذلك تم خلال هذه الدراسة دراسة مدى اتواجد جينات AME وهي *aac (٦′) Ie / aph (٢″)*، *aph (٣′) - IIIa ١*، *ant (٤′) - Ia ١* بالإضافة إلى جين *mecA* لـ بكتيريا المكورات العنقودية الذهبية المعزولة من عينات الجروح و الحروق، ١٢ عزلة (٢٤%) تحتوي على جين *mecA* بالإضافة إلى ذلك، ٣٢ عزلة (٦٤%) تحتوي على جين *aph(٣′)-IIIa ١* و ٦ عزلات (١٢%)

تحتوي على جين *aac(٦')Ie /aph(٢")*، في حين لم يلاحظ وجود الجين *ant(٤')-Ia ١* في أي من العينات.

ومن أجل حل مشكلة مقاومة البكتيريا للمضادات الحيوية، تم استخدام بعض النباتات الطبية لتحديد فعاليتها ضد البكتيريا. ومن بين هذه النباتات مستخلص الكركمين من نباتات *Curcuma longa* والبربرين من نبات *Berberis aristata*. وتشير النتيجة التي تم الحصول عليها إلى أن المبادئ النشطة بيولوجيا لهذه النباتات يمكن استخدامها بشكل خاص ضد هذه البكتيريا ذات الأصل السريري، حيث أثبت الكركمين فعاليته التثبيطية بتركيز ٢٠٠ ملغم / مل و M±SD (٢٥,٢٥ ± ٤,٠٨٩)، في حين كان للبربرين بتركيز ٢٥٠ ملغم / مل الفعالية التثبيطية حيث بلغت M±SD (٣١,٦٠ ± ٢,٥٠١) مع (قيمة $P = ٠,٠٥$).

بعد التأكد من فعالية المستخلصات النباتية كمضاد لبكتيريا المكورات العنقودية الذهبية تمت دراسة تأثيرها على الخلايا من خلال دراسة نشاط السمية الخلوية في مزارع الأنسجة باستخدام تقنية MTT. بالنسبة للكركمين على خلايا سرطان الثدي البشري (MCF-٧) في المختبر مقارنة بخلايا الكبد البشرية (WRL-٦٨)، وتأثير البربرين على خلايا سرطان الجلد (A٤٣١) وخلايا الخلايا الكيراتينية البشرية الطبيعية (HaCaT). أظهرت النتائج ارتفاع السمية الخلوية للكركمين ضد خلايا MCF-٧ عند التركيز (٤٠٠ ميكروغرام/مل). وبلغت حيوية الخلية (٣٧,٦٢±٣,٤٥١) وكانت قيمة $IC_{٥٠}$ (٢٦٩)، في حين كانت الفعالية الحيوية منخفضة جداً تجاه خلايا WRL-٦٨ عند التركيز (٤٠٠ ميكروغرام/مل)، وكانت (٧٧,٧٤±٣,٢١٠) وقيمة $IC_{٥٠}$. هو (٩١٩).

أما البربرين، فقد كانت سميته الخلوية ضد خلايا A٤٣١ عالية عند التركيز (٤٠٠ ميكروغرام/مل)، مع قابلية الخلية للنمو (٤٧,٩٦±٣,٩٦٦) وقيمة $IC_{٥٠}$ (٣٦٣)، بينما كانت سميته الخلوية منخفضة عند نفس التركيز تجاه خلايا HaCaT. (٧٤,٥٨±٦,٠٧٨) وقيمة $IC_{٥٠}$ عالية. (٩١٩) وهكذا نستنتج أن الكركمين والبربرين مصدران قيمان للأدوية المضادة للسرطان مع فعالية آمنة وانتقائية على الخلايا السرطانية مقارنة بالخلايا البشرية الطبيعية.

ولغرض دراسة التعبير الجيني لـ (*aph (٣') - IIIa ١*، *aac (٦') Ie / aph (٢")*، وجين mecA لسلالات بكتيريا المكورات العنقودية الذهبية المعزولة من عينات الجروح والحروق، تم عزل إجمالي الحمض النووي الريبي (RNA) من بكتيريا المكورات العنقودية الذهبية باستخدام مجموعة تنقية TRIzol وعكسها إلى cDNA قبل تقديمها لمزيد من التضخيم للتحقيق في

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التعبير الجيني لهذه الجينات باستخدام منهجية RT-qPCR قبل وبعد العلاج بالكركمين والبربرين. وصفت النتائج التعبير الجيني لـ *aac (٦′) Ie / aph* ، *aph (٣) - IIIa ١*، وجين *mecA* المرتبط بجينات الخاصة بالبكتيريا (١٦SrRNA)أظهرت الجينات انخفاض في مستوى التعبير الجيني، لجين *aac (٦′) Ie / aph (٢″)*، *aph (٣) - IIIa ١*، وكان جين *mecA* (٠,٦٩) و (٠,٩١) و (٠,٥٥) و (٠,٩٢) و (٠,٢٤) و (٠,٦٩) بعد معاملتها بالكركمين والبربرين على التوالي، وهذه النتيجة تعني أن معدل نمو البكتيريا والتعبير الجيني لجينات *aac* *(٦′) Ie / aph (٢″), aph (٣) - IIIa ١,* and *mecA* قد انخفض بعد المعاملة بالكركمين والبربرين.

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List of Abbreviations

Abbreviation	Complete term
Aap	Accumulation associated proteins
AK	Amikacin
ANT (4’)-I	Aminoglycoside- 4’-O-phosphoryltransferase I
ANT (6’)-I	Aminoglycoside- 6-O-nucleotidyltransferase I
APH(3’)-III	Aminoglycoside-3’-O-phosphoryltransferase III
AAC(6’)/APH(2’’)	Aminoglycoside-6’-N-acetyltransferase/2’’-O-phosphoryltransferase.
ANT (9’)-I	Aminoglycoside-9-O-nucleotidyltransferase I
AMEs	Aminoglycoside-modifying enzymes
BER	Berberine
β-hemolysin	Beta-hemolysin
CAT	Catalase
CWA	Cell wall-anchored proteins
JNK	c-Jun NH2-terminal kinase
CLSI	Clinical & Laboratory Standards Institute
Clf	Clumping factor
CFU	Colony Forming Unit
CA	Community-associated
CLSM	Confocal laser scanning microscopy
CUR	Curcumin
δ-hemolysin	Delta hemolysin
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DOS	Deoxystreptamine
DMSO	Dimethyl sulfoxide

ELISA	Enzyme-linked Immunosorbent Assay
A431	Epidermoid carcinoma cell line
EZH2	Expression of zeste homolog 2 gene
e DNA	external DNA
ECM	Extracellular Matrix
Embp	Extracellular matrix binding protein
EPS	Extracellular polymeric substance
EPS	Extracellular polymeric substance.
ERK	Extracellular signal-regulated kinase
FnBP	Fibrinogen binding protein
FnbpA	Fibronectin-binding proteins A
FnbpB	Fibronectin-binding proteins B
γ-hemolysin	Gamma hemolysin
CN	Gentamycin
GPx	Glutathione peroxidases
GR	Glutathione reductase
GN	Gram negative
GP	Gram positive
GPC	Gram-positive cocci
IC50	Half-maximal inhibitory concentration
HA	Healthcare associated
HaCaT	Immortalized human keratinocytes cell line
IE	Infectious Endocarditis
K	Kanamycin
MTT	Metabolic dye is3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
MRSA	Methicilin -Resistant *S. aureus*
MSSA	Methicilin –Sensitive *S. aureus*

MCF-7	Michigan Cancer Foundation-7
MSCRAMM	Microbial surface component recognising adhesive matrix molecule.
MAPK	Mitogen-activated protein kinase pathway
MHB	Mueller Hinton broth
MTT	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxy phenyl)-2-(4-sulphophenyl)-2H-tetrazolium
NCCLs	National Committee for Clinical Laboratory Standards
NEAT	Near iron transporter.
N	Neomycin
OM	Osteomyelitis
ORSA	Oxacillin- Resistant *S. aureus*
PVL	Panton Valentine Leukocidine
PBPs	Penicillin-binding proteins
PG	Peptidoglycan
PMS	Phenazine metho-sulphate
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesin .
PNAG	Poly-β(1-6)-Nacetylglucosamine
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative Polymerase chain reaction
RQ	Relative quantification
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute medium
SasG	*S. aureus* surface binding protein
SEM	Scanning electron microscopy
SSTI	Skin and Soft Tissue Infections

S. aureus	*Staphylococcus aureus*
S	Streptomycin
SOD	Superoxide dismutase
Spa	Surface binding protein A
WRL 68	The human hepatic cell line
Taq	*Thermus aquaticus*
TCPM	Tissue Culture Plate Method
TOB	Tobramycin
TSST-1	Toxic Shock Syndrome Toxin .
TEM	Transmission electron microscopy
TBE	Tris/Borate/EDTA
TSB	Tryptic soy broth
TRP1	Tyrosinase related protein 1
TRP2	Tyrosinase related protein 2
UV	Ultraviolet
VRSA	Vancomycin-Resistant *S. aureus*
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium

1.1. Introduction

The skin is the largest organ in the body and the first line of defense against invading pathogens such as viruses, fungi, parasites and bacteria , the skin serves as a physical barrier to prevent entry of bacteria into deeper layers of tissue and or dissemination to internal organ systems, keratinocytes form this important physical barrier (Maheswary *et al* ., 2021). Considering the potential of different types of Gram positive and Gram negative bacteria it becomes important to clinically suspect of skin infections (Tavares *et al.,* 2020). Burn injury is one of the most common types of trauma that requires urgent medical attention (Jeschke *et al* ., 2020). Normal protective defense mechanisms of the skin are lost after a burn injury, resulting in rapid colonization of the wound surface, Initially, Gram-positive organisms derived from skin commensals colonize the wound bed, followed later by Gram-negative organisms and yeasts (Ersanli *et al* ., 2023).

S. aureus is a Gram positive commensal opportunistic and lifethreating pathogen bacterium. In 1880, for the first time, Alexander Ogston reported the isolation of *S. aureus* from a surgical wound infection. Also in the same year, Louis Pasteur, showed abscesses production in animals injected with pus from human staphylococcal infections (Somerville, 2016). The primary natural habitats of this bacterium are the surface of skin and mucosae of humans. The squamous epithelium of the anterior nares is the preferred niche especially in adults (Mulcahy and McLoughlin, 2016). *S. aureus* is one of the most common causes of nosocomial bacteremia, blood stream infections, hospital-acquired pneumonia, surgical site infections, skin and soft tissue infections (SSTI), infectious endocarditis (IE) osteomyelitis (OM), device

related infections and breast implant infections. The mortality rate of S. aureus bacteremia is around 20–30% (Denis, 2017; Ansari *et al.*, 2019).

The density of *S. aureus* in persistent carriers in the nose is higher than intermittent carriers with a single genotype and these persistent carriages can cause increased risk for nosocomial infections such as bacteremia, dialysis infections, and surgical site infections (Denis, 2017). *S. aureus* strains have two states; planktonic and biofilm state with different specifications (Parastan *et al.*, 2020). Biofilms are sessile microbial communities that rest in an extracellular matrix (ECM) which may be composed of polysaccharide, protein or external DNA (e DNA) (Gupta *et al.*, 2021).

Antibiotic resistance was considered a major public health issue with reports of an increasing trend of resistance in clinical isolates (Minarini *et al.*, 2020).Recently the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics have reaffirmed the need to probe the antimicrobial activity of medicinal plants (Taylor 2013). Aminoglycosides are bactericidal and broad-spectrum antibiotics often used along with either β-lactam or glycopeptides for treatment of serious infections caused by Gram-positive cocci (GPC), particularly *Staphylococcus aureus* and *Staphylococcus epidermidis* (Liakopoulos *et al.*, 2011). Resistance to aminoglycosids in *S. aureus* may result from various mechanisms such as: (1) Synthesis of transferases (acetyltransferases, phosphotransferases, nucleotidyltransferases) that modify the aminoglycoside molecule (Emaneini *et al.*, 2009*)*.

The only aminoglycoside not modified by most enzymes (except AAC(2′)-Ia,b,c) is plazomicin, but it is currently not recommended for the treatment of *S. aureus* infections (Clark *et al.,* 2020). (2) Lack of enzymes responsible for active transport of aminoglycosides into the bacterial cell (Melter and Radojevič 2010).

The most common mechanism of resistance to aminoglycosides in *S. aureus* is the synthesis of enzymes of the transferase group (Szymanek *et al.,* 2018).The most significant are: the two-domain acetyltransferase /phosphotransferase AAC(6′)-Ie/APH(2”)-Ia, encoded by the aacA-aphD gene and causing resistance to gentamicin, tobramycin, kanamycin, amikacin and netilmicin, ANT(4′)-Ia nucleotidyltransferase encoded by aadD gene and causing resistance to tobramycin, kanamycin, neomycin and APH(3′)-IIIa phosphotransferase encoded by aph(3′)-IIIa gene causing resistance to kanamycin, neomycin, and lividomycin An APH(3′)-III phosphotransferase conditioning resistance to kanamycin and neomycin, encoded by the aphA-3 gene (another name for the aph(3′)-IIIa gene), has also been described in vancomycin-resistant *S. aureus* (VRSA) (Ida *et al.,* 2001).

Medicinal herbs could be the best source for various medicines (Wolf *et al.,* 2021). Due to their different therapeutic properties, medicinal herbs have been considered by many researchers worldwide (Zhang *et al.,* 2016). In modern medicine, several studies have been conducted to find the potential effects of various extracts of medicinal herbs that have a pivotal role in the health of people and animals (Abd El-Hack *et al.,* 2021 and El-Saadony *et al.,* 2022).

Plant-based drugs may be much more appropriate in biochemical terms when compared to synthetic drugs. However, modern medicine does essentially support natural products for medicinal uses (Yaqoob *et al.*, 2021), such as growth inhibitors of some tumors (Zhang *et al.*, 2021). Over the few current decades, considerable studies have been conducted on curcumin due to its beneficial health properties, including potent antioxidant properties (Guo *et al.*, 2018), antimicrobial (Trigo-Gutierrez *et al.*, 2021) and anticancer effects (Abd Wahab *et al.*, 2020).

Berberine (BER) is an isoquinoline quaternary alkaloid (a 5, 6-dihydrodibenzo [a, g] quinolizinium derivative) employed in traditional Chinese and Indian medicine for centuries (Behl *et al.*, 2022). It has anti-inflammatory and antimicrobial properties, anti-diabetic and antioxidant effects and multiple pharmacological properties. BER has further been shown to have antitumor effects on many cancer cell lines, including leucocytes, liver, lung, stomach, colon, skin, oral, esophageal, brain, bone, breast, and genital cancer cells (Guamán *et al.*, 2014).

Cell viability and cytotoxicity tests assess cytotoxic effects and acute systemic toxicity to ensure safety and effectiveness before clinical use. Cell viability, indicating the number of healthy cells in a sample, is determined through various assays that measure live-to-dead cell ratios. Cytotoxicity measures a substance's potential for cell damage or death, and is evaluated through numerous assay methods based on different cell functions (Sukumaran *et al.*, 2023).

1.2. Aims of study

Because of the problem of bacterial resistance to antibiotics in a large way and the resulting compensations, the current study aimed to investigate the relationship between the antibiotic resistance patterns and aminoglycosides resistance gene in *S. aureus* isolated from wounds and burns infections , and also ,the possibility of using medicinal plants as curcumin and berberine with aminoglycosides to eliminate bacteria and solve the problem of bacterial resistance to antibiotics.

For the purpose of achieving the objective of the current study, the study included:

1. Isolation and identification of *S. aureus* bacteria from burns and wounds.
2. Aminoglycosides antibiotic susceptibility test to identify resistant isolates.
3. Detection biofilm production test.
4. Detection of resistance genes to aminoglycosides by PCR technique.
5. Determination effect of medicinal plant (curcumin and berberine) extract on resistant *S. aureus* isolates and on (MCF-7, WRL68, A431 and HaCaT) cell lines.
6. Gene expression of *mecA* , *aph(3')-IIIa1* ,*aac(6')Ie/aph(2'')* and *ant(4')-Ia1* gene for isolates of *S. aureus* affected by curcumin and berberine .

2. Literature review

2.1. Background

The skin is the first and largest barrier of the human body. It covers the human organism and ensures a constant dialogue with the external environment full of exogenous factors, such as foreign pathogens, ultraviolet (UV) radiation, and allergenic and chemical irritants. Therefore, through evolution, a dynamic cutaneous ecosystem has been developed in order to protect the host from undesirable insults and aggressions (Peck , 2011).

Burn wound infections are one of the most important and potentially serious complications that occur in the acute period following injury. These wounds are subsequently colonized by microorganisms, including Gram-positive bacteria, Gram-negative bacteria and Yeasts, which derived from the host's normal flora (gastrointestinal flora, upper respiratory flora) and from the hospital environment (Mohammed, 2007).

Thermal damage to the protective skin barrier and the underlying exaggerated pathophysiological response characterized by persistent hormonal disturbances, hyperinflammation, hypercatabolism, hypovolaemia, and immunosuppression make the burn patient very susceptible to infection and advanced morbidity related to that infection (Dinsdale *et al.*, 2020).

Burn wound surfaces are initially sterile and subsequently become colonized with Gram-positive organisms present within hair follicles and sweat glands (Sharma, 2007). Following admission, burn wounds can become colonized from exogenous sources or through translocation of Gram-negative bacteria usually from the patient's gastrointestinal system (Laurie *et al.*, 2011).

Interventions such as early excision have been shown to reduce the risk of invasive sepsis from burn wound colonization (Barret & Herndon 2003). Early grafting, topical and systemic antimicrobials, and strict infection control policies have also changed the landscape of burn wound microbiology, these interventions have become essential parts of burn care over time, almost across the board. Other important factors that influence burn wound infection include local nosocomial pathogens and duration of hospitalization (Cato *et al.*, 2023).

Furthermore, the incidence of burn wound infection appears to be correlated with both the depth and the size of wound. Moreover, the longer the wound remains open, the higher the chances on infection, previous studies have shown that there are several bacterial species which are able to readily infect burn wounds. *S. aureus* and *P. aeruginosa* have been found to be the most common species. Interestingly, the variation in bacterial flora and the colonization rate changes over time after the initial infection (Taneja *et al.*, 2013).

Improved medical treatment and infection control has already reduced the death rate of burn patients significantly. The amount of death-related burns has been halved within the past 40 years (Laurie *et al.*, 2011). Moreover, bacterial infection can be controlled by the use of antibiotics. A downside of the use of antibiotics is that some pathogenic strains of bacteria may become multidrug-resistant (Keen *et al.*, 2010).

The organisms responsible for infections in patients who suffer from burn injuries may be endogenous or exogenous which can change over time in the individual patient (Kharre *et al.*, 2020). One of the most common burn wound pathogens is *S. aureus. S. aureus* is both a human commensal and a frequent cause of infections ranging from mild to life-threatening diseases. Colonization with *S. aureus* has been associated with delayed wound healing, increased need for surgical interventions,

and prolonged length of stay at burn centers (Kooistra-Smid *et al.*, 2009). Microorganism is transmitted to the burn wound surfaces of admitted patients by the hands of medical personnel, by fomites, and from patient's skin surface and nose and during intervention (Kharre *et al.*, 2020).

2.2. Genus Staphylococcus

About 36 species and several subspecies are recognized in the genus *Staphylococcus* until 2006. The three main species of clinical importance are; (1) *S. aureus*, which causes infections in almost every organ and tissue of the human body with the fact that the most commonly affected part of the body due to *S. aureus* infection is the skin (Daum, 2007), (2) *S. epidermidis* that causes infections associated with indwelling medical devices (Vadyvaloo and Otto, 2005) and (3) *S. saprophyticus* wich causes urinary tract infections commonly associated with young girls (Horowitz and Cohen,2007). It plays a significant role in causing infections in both hospitals and community ranging between simple to life threating infections (Al-sa'ady , 2015; Oliveira *et al.*, 2018)

2.2.1. Cellular characteristics

S. aureus is a gram-positive bacterium, spherical in shape, and sized between 0.5.-1.5 µm in diameter (Gnanamani *et al.*, 2017; Bitrus *et al.*, 2018). It is non-motile and non-spore forming, *S. aureus* appears bluish/purple by Gram staining (Taylor and Unakal 2017) ,and can be observed microscopically as a single coccus, in pairs, or in grape-like clusters (Gnanamani *et al.*, 2017). It is a facultative anaerobe that obtains energy for growth by aerobic respiration or fermentation. *S. aureus* divides by binary fission; its cell division occurs at different planes and its optimum growth occurs at temperatures ranging between 18-40 °C (Taylor and Unakal 2017).

The organism causes infections by owing different virulent genes that encode different virulent factors such as toxins and enzymes, among others. Further, the virulence of *S. aureus* has risen with existence of antibiotics resistance strains such as Methicillin resistant S. aureus (MRSA) and Vancomycin resistance *S. aureus* (VRSA) (Turner *et al.*, 2019).The resistance strains increased the challenge in treating the infections caused by them. The circulation of these strains in health care settings and community changed the epidemiology of their spread. Using preventive control measures are critical in controlling *S. aureus* infections (Oliveira *et al.*, 2018).

2.2.2. Colony morphology

S. aureus colonies are characterized by their large, smooth, and elevated appearance with a golden yellow color (Bitrus *et al.*, 2018). The yellow color is the result of staphyloxanthin (a carotenoid) produced by the bacteria, which covers and protects the microorganism from phagocytosis (Thomer *et al.*, 2016). Usually the microorganism causes hemolysis on enriched agar (blood agar with 5% sheep or horse blood), producing zones around the colonies ,this hemolysis is due to the production of different kinds of enzymes called hemolysins (Gnanamani *et al.*, 2017) . *S. aureus* is grown on selective media, such as mannitol salt agar containing 7.5-10% sodium chloride, as *S. aureus* is salt tolerant (Taylor and Unakal 2017).

The pink color of medium shifts to yellow during the microorganism‘s fermentation of mannitol sugar, which yields an acid and changes the color of the medium. This can be utilized to distinguish *S. aureus* from *S. epidermidis*, a non-mannitol fermenter (Kumara *et al.*, 2017).

These illnesses may range from simple skin and soft tissue infections to more serious and life threatening conditions such as blood infections (bacteraemia/septicaemia) (Kobayashi *et al.,* 2015).

2.2.3. Virulence factors

S. aureus causes a broad spectrum of infections as a virulent pathogen. Its virulence is attributed to several various virulence factors that help in the different steps of infection (Zecconi and Scali 2013). The bacterial virulence factors act as a synergistic group have many functions such as adhesion to eukaryotic membranes, opsonophagocytosis resistance, eukaryotic cell lysis , and trigger the production of a cascade of host immune modulating molecules. So, it is difficult to sort out the role that individual virulence factors play in pathogenesis. The virulence factors can be categorized into adherence factors, exoproteins, biofilm formation, and staphylococcal pigments. Table (2-1) focuses on understanding the virulence factors in *S. aureus* and their role in infectious diseases (Stephens 2007).

In Iraq, a vast number of Iraqi published reports emphasized that *S. aureus*, isolated from different sources, has a broad spectrum of virulence factors responsible for various human and animal diseases and associated with asymptomatic colonization of normal humans (Hanon 2016 ; Saleem 2017).

2.2.4. Pathogenesis

The pathogenicity of *S.aureus* infections is related to various bacterial surface components (capsular polysaccharide and protein A), including those recognizing adhesive matrix molecules (clumping factor and fibro nectin binding protein) and to extracellular enzymes and toxins

(coagulase, staphylokinase, hyaluronidase, haemolysins, enterotoxins, toxic shock syndrome toxin, and Panton Valentine Leukocidine (PVL) (Sinha *et al.,* 2000).

The *coa* gene is one of the most important virulence factors For *S.aureus* Expression of this gene is thought to enhance bacterial growth and promote infection in the face of host defense mechanisms, such as phagocytosis (Karahan, and Cetinkaya 2007).

The adherence of *S. aureus* to a host tissue is an important step in pathogenesis as well as in colonization. Surface proteins, such as protein A, clumping factors, fibronectin-binding proteins, and collagen-binding proteins, can adhere to extracellular matrix components of the host (Kouidhi *et al.,* 2010). Almost all strains produce and secrete enzymes and exotoxins, including hemolysins (alpha, beta, gamma, and delta), proteases, lipases, nucleases, hyalonuridase, and collagenase (Dinges *et al*., 2000). Alphahemolysin (or *alpha-toxin*) (α-toxin) is dermonecrotic, neurotoxic, and lysis mammalian cells especially red blood cells by forming a pore in the targetmembrane (Chanda *et al*., 2010).

Beta-hemolysin (β-hemolysin), wich acts as sphingomyelinase, gamma hemolysin (γ-hemolysin), has leucocytolytic activity. It has been suggested that delta-hemolysin (δ-hemolysin) has surfactant or channel forming properties (Dinges *et al*., 2000).

S. aureus must produce components capable of attaching to cells or tissues, factors that decrease phagocytosis in order to escape the host immune system, and to modify proteases, exotoxins, and enzymes, causing tissue damage, and thus, allowing the dissemination of *S. aureus*.(Prevost *et al*., 2001). All these virulent factors show in table (2-1).

Table (2-1): Most Common Virulence Factors in *S. aureus* (Stephens 2007).

Category	Virulence factor
Adherence Factors (Adhesions): surface proteins and antigens	Staphylococcal protein A (SpA) Fibronectin-binding proteins A and B (FnbpA and FnbpB). Collagen-binding protein Clumping factor (Clf) A and B proteins Capsule Teichoic acid
Exoproteins	Enzymes Toxins
Enzymes	Catalase Coagulase Lipase Proteases Phosphatase Hyaluronidase Staphylokinase Nuclease Beta-Lactamase
Toxins	α-Toxin (α-Hemolysin) β-Toxin (β-Hemolysin) δ-Toxin Panton-Valentine Leukocidin(PVL) Leukocidin Staphylococcal Enterotoxin Exfoliative Toxins Toxic Shock Syndrome Toxin (TSST-1)
Staphylococcal	Staphyloxanthin Pigments

2.2.5. *Staphylococcus aureus* biofilm

S. aureus secretes an extracellular polymeric substance (EPS), known as biofilm, that helps the microbe to resist and minimise the effect of

antibacterial drugs (Kaplan *et al.,* 2018). Similar to any other bacterial biofilm, a *S. aureus* biofilm also has two distinct components, i.e., water (about 97%) and the organic matter which includes EPS and microcolonies (Nazir *et al.*, 2019).

The EPS constitutes about 50 to 90% of the total organic matter of a biofilm and is a complex of different polymeric substances, such as extracellular DNA (eDNA), proteins and polysaccharides(Idrees *et al.*, 2020) .The remaining portion, 10–25%, consists of microcolonies (Nazir *et al.,* 2019). In *S. aureus* biofilm, the major component of EPS is the polysaccharide intercellular adhesin (PIA) (Reffuveille *et al.,* 2017).

The polysaccharide component of EPS has been given the name PIA due its function, i.e., intercellular adhesion of bacterial cells, and poly-β(1-6)-Nacetylglucosamine (PNAG), due to its chemical composition. PIA are cationic in nature and play a significant role in colonisation, biofilm formation and biofilm-related infections, immune evasion, resistance to antimicrobials and phagocytosis (Nguyen *et al.*, 2020).

S. aureus EPS also contains a range of proteins including accumulation associated proteins (Aap), surface binding protein A (Spa), fibrinogen binding protein (FnBP) A and B, extracellular matrix binding protein (Embp), amyloid fibres and *S. aureus* surface binding protein (SasG) (Dutta *et al.,* 2016). These S. aureus proteins perform different functions. For example, accumulation associated protein Aap interacts with PIA and plays a role in biofilm maturation (Reffuveille *et al.,* 2017).

CWA proteins facilitate adhesion to EPS, to host surface, and their interaction with CWA proteins on adjacent cells contributes to the accumulation of biofilm (Speziale *et al.,* 2014).

Similarly, amyloid fibres act as a scaffold that keeps *S. aureus* cells anchored to the biofilm matrix and thus maintain the stability of the biofilm (Taglialegna *et al.,* 2016; Idrees *et al.,* 2020).

Alongside PIA and EPS proteins, the third important component of *S. aureus* biofilm EPS is eDNA. eDNA has been reported to be involved in irreversible attachment, horizontal gene transfer, maintaining biofilm integrity, antimicrobial resistance and host immune system evasion (Miao *et al.,* 2019).

2.2.6. Biofilm Formation

2.2.6.1. Stages

The formation of biofilm proceeds through four different stages (Landini *et al.,* 2010), which are

1. Attachment of planktonic cells to the surface (either a biotic host or any abiotic surface);
2. Colonisation and biofilm formation;
3. Biofilm maturation;
4. Biofilm dispersal. Biofilm formation in *S. aureus* is initiated when free floating, planktonic cells attach to the available surface and start colonising (Petrova *et al.*, 2012).

S. aureus adherence to a surface is influenced by hydrophobic and hydrophilic interactions between the S. aureus cell surface and any biotic or abiotic surface (Krasowska and Sigler, 2014; Maikranz *et al.,* 2020) . It has been found that the *S. aureus* cell surface adheres to hydrophobic surfaces by the help of many weakly binding macromolecules, while its adherence to hydrophilic surfaces involves fewer but stronger binding macromolecules (Otto 2018).

The formation of micro colonies is followed by the formation of an extracellular polymeric substance (EPS) that develops into a fully matured biofilm (Landini *et al.*, 2010).

Once the biofilm is fully matured, the bacterial cells residing inside it release certain chemicals, i.e., D amino acids and EPS-degrading enzymes such as alginate lyase, to break and disperse the biofilm (Kostakioti *et al.*, 2013). These planktonic cells are ready to either recolonise the same site or attach to a different site and repeat the process to form a new biofilm (Donlan 2002).

Figure (2-1) depicts different stages involved in the formation of a bacterial biofilm. *S. aureus* cells that are encased and protected by biofilms show different phenotypic characters compared to cells in their planktonic form. Biofilm-associated *S. aureus* cells are more resistant to antibiotics and exhibit differences in cell size and growth, gene expression and protein production, compared to their free living counterparts (Otto , 2018).

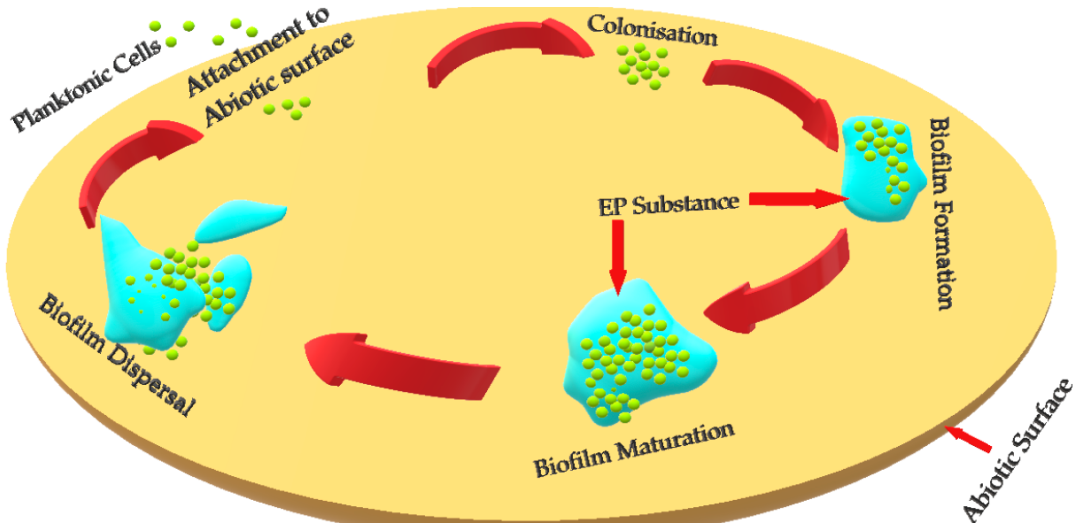


Figure (2-1) Depiction of *S.aureus* biofilm formation on an abiotic surface. Basic concept has been adopted from (Idrees *et al.*, 2020)

2.2.6.2. Techniques and Strategies Used in Studying *S. aureus* Biofilm

Microbiologists adopt different approaches based on different techniques to study and evaluate *S. aureus* biofilm. These techniques are either direct evaluation of biofilm, based on the measurement of thickness of a biofilm, or indirect techniques of measurement where a biofilm is quantified in terms of studying its different constituents or the activity of the bacterial cells within the biofilm (Idrees *et al.*, 2021). These techniques are discussed in the following sections.

2.2.6.2.1. Direct Observation Techniques

Direct observation of the intricacy and changing aspects of biofilms can be studied by biofilm optical imaging technology, comprising light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). These methods are employed to examine the presence of biofilm and visualize its 3D structure (Roy *et al.*, 2018).

2.2.6.2.2. Indirect Observational Techniques

These include the following:

2.2.6.2.2.1. Tube Method

This is a qualitative technique described by Christensen *et al.*, for the detection of biofilms. A solution of 1% crystal violet was used to stain the biofilm and observe the film around the wall and bottom of the test tube to confirm a biofilm producer. The extent of biofilm formed was recorded as 1: weak/none, 2: moderate and 3: high/strong. Ring formation at the liquid–air interface was not considered indicative of slime production (Taj *et al.*, 2012).

This technique is frequently subjected to analytical restrictions and is incompetent for detecting bacterial adherence precisely. Therefore, to overcome these shortcomings, the following method can be adapted to modern approaches to obtain precise findings.

2.2.6.2.2.2. Congo Red Agar Method

The Congo red agar screening technique is used to detect whether the *Staphylococcal* isolates are biofilm producers. A positive result is shown by black colonies with a dry crystalline consistency. Weak slime producers are indicated as pink; however, darkening is seen at the centres of colonies occasionally. The blackening of the colonies devoid of dry crystalline morphology suggested moderate biofilm procedures(Mathur *et al.*, 2006).

2.2.6.2.2.3. Detection of Biofilm Production by Microtiter Plate Assay

The microtiter plate assay is a standard quantitative technique to detect biofilm production. The duration of incubation can be modified as needed, and the medium of choice for evaluating the biofilm production is TSB or MHB with 1% glucose. The technique was adapted for better quantification of the biofilms. Optical density (OD) was determined with a microplate reader at a wavelength of 570 nm (OD 570 nm)(O'Toole , 2011; Sanchez *et al.*, 2013).

2.3. Antibiotic Resistance

2.3.1. Overview

An antimicrobial refers to a substance that kill or inhibit the growth of microorganisms (Zhang *et al.*, 2010).Since the discovery of antimicrobial drugs in the 1923,many infectious diseases have been overcome. Typically, antimicrobials kill bacteria by binding to some vital

compounds of bacterial metabolism, thereby inhibiting the synthesis of functional biomolecules or impeding normal cellular activities (Coates *et al.*, 2002). Antibiotics work in a variety of ways. Some antimicrobial agents inhibit bacterial cell wall synthesis.

These agents include β-lactam compounds, such as penicillins (penicillin G, ampicillin and methicillin), cephalosporins and carbapenems, as well as mono lactams and β-lactamase inhibitors. β-lactams inhibit the final stage of murein synthesis. This, by some undetermined mechanism, triggers murein hydrolases to lyse the cell. A related group of antibiotics that prevent a different step in cell wall synthesis is the glycopeptides, vancomycin and teicoplanin (Fisher *et al.*, 2020).

In bacteria, resistance evolves through naturally occurring genetic mutation and the process of natural selection. The organism in question may develop the ability to destroy the antibiotic or to grow in its presence (Holcomb *et al*., 2008). Methicillin (β-lactamase-resistant penicillin) and its derivatives become the drug of choice for the treatment of infections caused by *S. aureus*. However, prolonged hospitalization and antibiotic therapy especially with β-lactam antibiotics, predispose patients to the acquisition of methicillin / oxacillin- resistant *S. aureus* (**MRSA**/**ORSA**) (Goto *et al*., 2009; Mattner *et al*.,2010). Methicillin-resistant *S. aureus* (MRSA) is a strain of *S. aureus* which, by definition, is resistant to the semi- synthetic penicillins (methicillin, nafcillin, and oxacillin) (Onanuga and Temedie, 2011).

S. aureus isolates from intensive care units across the country and from blood culture isolates worldwide are increasingly resistant to a greater number of antimicrobial agents (Couto *et al* .,1995).

Inevitably, this has left fewer effective bactericidal antibiotics to treat these often life-threatening infections . As rapidly as new antibiotics are introduced, staphylococci have developed efficient mechanisms to neutralize them . *S. aureus* isolates from intensive care units across the country and from blood culture isolates worldwide are increasingly resistant to a greater number of antimicrobial agents (Couto *et al.*, 1995). Table (2-2).

2.3.2. Mechanisms of Antimicrobial Resistance in Bacteria

Mechanisms of antimicrobial resistance come in four general forms: (Quale *et al* ., 2003), figure (2-2)

1. Enzymes that destroy or modify the antimicrobial substrate.
2. Target site alteration like alteration of DNA gyrase, a target of fluoroquinolones.
3. By pass pathways that substitute for a metabolic pathway.
4. Barrier to penetration or efflux pumps that exclude the agent.

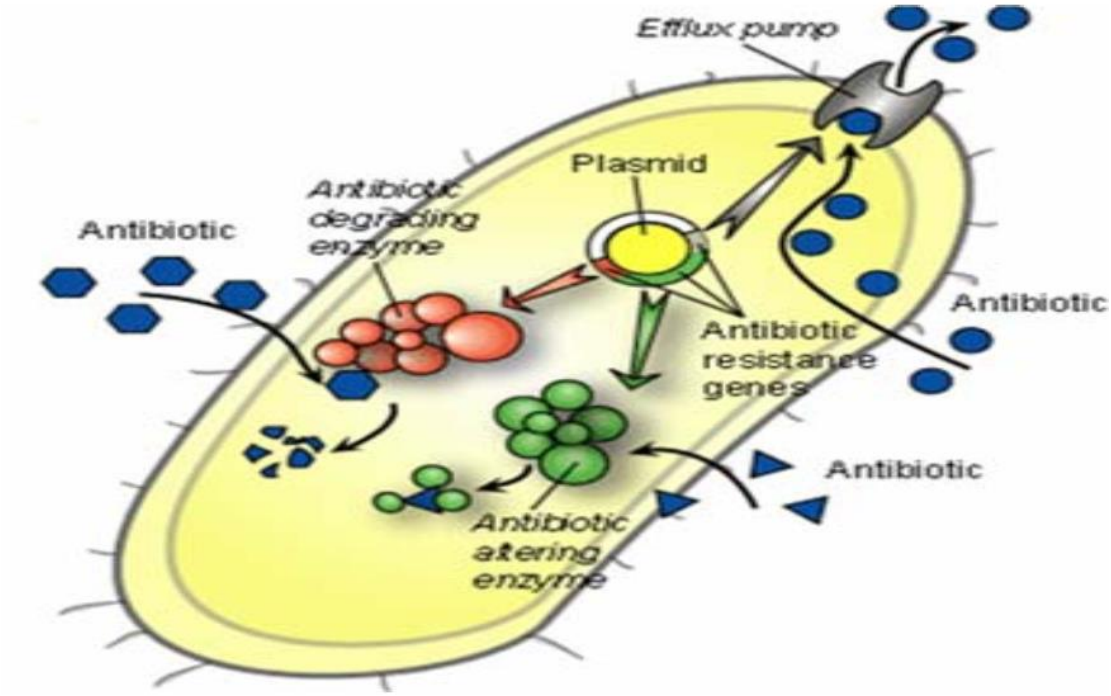


Figure (2-2): Mechanisms of antimicrobal resistance (Alalem, 2008).

S. aureus is one of the most significant pathogens in terms of antibiotic resistance, as it has been able to develop resistance mechanisms to nearly all antibiotics used against it. Indeed, from the early 1940s, when penicillin resistance in *S. aureus* was initially described, *S. aureus* has steadily acquired new mechanisms of resistance, allowing it to become resistant to all beta-lactams, tetracyclines, aminoglycosides, fluoroquinolones, clindamycin, trimethoprim-sulfamethoxazole, vancomycin, daptomycin and linezolid (Foster 2017).

2.3.3. Staphylococcal resistance to aminoglycoside antibiotics

Aminoglycosides are grouped into 4,6-disubstituted 2-deoxystreptamine (DOS), 4,5- disubstituted DOS, and 4-monosubstituted DOS based on their chemical structures Representative 4,6-disubstituted DOS agents include gentamicin, tobramycin, and amikacin, which are widely used as intravenous or nebulized formulations for the treatment of infections caused by gram-negative bacteria (usually in combination with a β-lactam agent), gram-positive bacteria (for synergistic activity with a β-lactam or peptidoglycan), and atypical mycobacteria (again in combination with other active agents). 4,5-Disubstituted DOS agents, represented by neomycin, are limited in their utility by toxicity and are administered either orally or topically but not intravenously. Monosubstituted DOS agents are represented by apramycin, which is used in veterinary medicine (Doi *et al.,* 2016) .

Aminoglycosides are bactericidal antibiotics that inhibit protein synthesis by interfering with the 30S subunit of the ribosome (Mlynarczyk *et al.,* 2022). Due to their ability to induce errors in mRNA translation. While normally the bacterial ribosome may have an error rate of 1–1000 to 1–10,000 amino acids, aminoglycosides increase the error

rate to 1–100 amino acids, which translates to the average protein containing approximately 3 mistakes. This has lethal consequences for the bacterium, especially in the case that the disrupted proteins constitute membrane proteins (Walsh , 2016). In *S. aureus*, aminoglycoside resistance is mediated through enzymatic inactivation, specifically through enzymes that acetylate and phosphorylate aminoglycosides (Kelmani Chandrakanth *et al.*, 2008).

2.3.4. Antibiotic resistance genes & susceptibility patterns in staphylococci

S.aureus is one of the most important serious opportunistic human pathogens involved in nosocomial infections (Stefani *et al.*, 2012 ;van Bijnen *et al.*, 2014) . Resistance to methicillin primarily derives from acquisition of the mecA gene, which encodes a modified penicillin-binding protein (PBP2a) with low affinity for b-lactams (Stefani *et al.*, 2012). In recent years, the emergence of strains resistant to methicillin and other antimicrobial agents has become a major concern worldwide, especially in the hospital environment, where there is a higher mortality rate due to systemic methicillin-resistant S. aureus (MRSA) infections (Dulon *et al.*, 2011).

Resistance to aminoglycosids in *S. aureus* may result from various mechanisms such as: (1) Synthesis of transferases (acetyltransferases, phosphotransferases, nucleotidyltransferases) that modify the aminoglycoside molecule (Emaneini *et al.*, 2009), (2) Lack of enzymes responsible for active transport of aminoglycosides into the bacterial cell (Melter 2010). Aminoglycosides are one of the classes of antibiotics that play an important role in the treatment of staphylococcal infections. These are often used synergistically in combination with either beta-

lactam or glycopeptides, especially for the treatment of complicated staphylococcal Infections (Ida *et al.,* 2001). The main mechanism of resistance to aminoglycosides is the inactivation of antibiotics by aminoglycoside-modifying enzymes (AMEs) that are encoded by genetic elements(Hauschild *et al.*, 2008).

In general resistance to aminoglycosides are predominantly mediated by five classes of Aminoglycoside modifying enzymes (AMEs) which are major factors responsible for resistance to aminoglycoside in staphylococci included **(1)** aminoglycoside-6'-N-acetyltransferase/2''-O-phosphoryltransferase ([AAC (6')/APH(2'')] encoded by the *aac(6')/ aph(2'')* gene; **(2)** aminoglycoside-3'-O-phosphoryltransferase III [APH(3')-III] encoded by *aph (3')-IIIa* gene; **(3)** aminoglycoside- 4'-O-phosphoryltransferase I [ANT (4')-I] encoded by *ant(4')-Ia* gene; aminoglycoside-9-O-nucleotidyltransferase I [ANT (9)-I] encoded by the *ant (6)-I*, and **(4)** aminoglycoside- 6-O-nucleotidyltransferase I [ANT (6)-I] encoded by the *ant (6)-I* gene .In staphylococcal strains, the most commonly found AME is *aac(6')/aph(2'')*. The bifunctional enzyme *aac(6')/ aph(2'')* is encoded by the *aac(6')/aph(2'')* gene. In addition, APH(3')-III is encoded by *aph(3')- IIIa* gene and the ANT(4')-I by *ant(4')-Ia* gene, are also found in staphylococcal isolates (Ida *et al.*, 2001 ; Sekiguchi *et al.*, 2004 ; Yadegar *et al.*, 2009).

Clinically, the most common AMEs in staphylococci are ANT (4')-I, AAC (6')/APH(2'') and APH (3')- III, which modify aminoglycosides of therapeutic significance, including tobramycin, gentamicin and kanamycin, respectively(Ramirez and Tolmasky 2010) . The accurate and rapid diagnosis of antibiotic resistance genes in the treatment of staphylococcal infections is extremely important in preventing the spread of infections. PCR-based molecular methods are often preferred for

determination of antibiotic resistance genes (Woodford and Sundsfjord 2005).

2.4. Antibacterial Activity of medical plants Extract Curcumin and Berberine against *S. aureus*.

2.4.1. Overview

The extensive, inappropriate, irregular, and indiscriminate uses of antibiotics have resulted in the emergence of antimicrobial resistance, making many currently available medications ineffective (World Health Organization 2014; Baym *et al.*, 2016). Therefore, there is an increasing demand to develop new antimicrobial agents that are able to decrease the use of antibiotics and to face resistance development. This has directed researchers to isolate and identify new bioactive chemicals from plants to act against microbial resistance (Khameneh *et al.*, 2016;, Tortorella *et al.*, 2018) . Medicinal plants are rich in a wide variety of chemical compounds, which have been found in vitro to have antimicrobial activities (Lewis and Ausubel, 2006). Natural antimicrobial agents can act alone or in combination with antibiotics to enhance antimicrobial activity against a wide range of microbes (Bazzaz *et al.*, 2018). Extracts isolated from medicinal plants have been reported exhibit various biological activities such as antimicrobial, anti-inflammatory, and antioxidant activities (Mehta *et al.*, 2001).

The antimicrobial compounds from medicinal plants may inhibit the growth of bacteria, fungi, viruses, and protozoa by different mechanisms than those of presently used antimicrobials and may have a significant clinical value in the treatment of resistant microbial strains (Shankar *et al.*, 2010).

2.4.1.1. Curcumin

Medicinal plants are important source for the verification of pharmacological effects and can be natural composite sources that act as new anti-infectious agents (Ushimaru *et al.,* 2007; Hussain *et al.,* 2016 ;Ali *et al.*, 2017; Amin *et al.*, 2017). Curcumin, a yellow crystalline polyphenol with low molecular weight, is extracted from rhizome of turmeric. Turmeric belongs to the perennial herb named *Curcuma longa L.* which is prevalent in tropical and subtropical regions, mostly in India, South East Asia and China. India is the first producer, consumer and exporter of *Curcuma longa* in the world (Sahne *et al.*, 2016).

Curcumin has wide range of applications as a dietary food ingredient, dying agent, therapeutic agent and medicament in different diseases. Curcumin has various pharmacological effects including antioxidant activities (Tajik *et al.,* 2007).

Curcumin (also called dipheulolymethane) is a symmetric molecule that gives the yellow color, the chemical definition of curcumin is [1,7-bis (4-hydroxy-3methoxyphenyl)-hepta-1,6-diene-3,5 dione] and it is chemical formula is (Oglah *et al.*, 2020). It is used to treats several injuries, skin and eye infections, acne, autoimmune diseases and neurological disease (Giordana and Tommonaro, 2019). Boroumand *et al.* (2018) stated that curcumin have a cholesterol-lowering anti-diabetic, antiinflammatory, anticancer and antioxidant properties. Also, it has a potent antioxidant action through free radical-scavenging activity (Al-Samydai, 2018).

On the other hand, it is a physiologically active component that has antimicrobial properties and it is effective against *S. aureus, Salmonella paratyphi, Mycobacterium tuberculosis and Trichophytum gypseum* (Hewlings and Kalman, 2017).

Several investigations on the antimicrobial activity of herbal and plant extracts against drug-resistant pathogens have been carried out to improve the safety of medicinal medications. Alternative sources of natural bioactive chemicals from medicinal plants are being studied to replace the traditional antibiotics and synthesize antimicrobial compounds (Liu *et al.,* 2019). More than 80% of people worldwide, mostly in developing countries, employ numerous plant extracts and active compounds as traditional medicine in conventional pharmaceuticals, according to the World Health Organization (Kirbağ *et al.*, 2009).

Curcuma longa L. (Turmeric) belongs to the Zingiberaceae family botanically. Turmeric has antibacterial, antifungal, antiviral, anti-aging, antimalarial, anticancer, anti- Alzheimer's disease, antioxidant, and anti-inflammatory properties (Boroumand *et al.,* 2018).

The curcumin and the oil fraction inhibit the growth of many bacteria such as *E. coli, Streptococcus, Salmonella Typhimurium, Staphylococcus, Yersinia enterocolitica, Bacillus subtilis* and *B. cereus* (Kai Kai *et al.,* 2020).

Turmeric has healthy influence on digestive system and it also enhances the mucin secretion in the digestive tract. In classical literature several actions of turmeric have been specified like antibacterial, antithelmintic, anticancer, anti-parasitic, antiseptic, anti-oxidative, anti-inflammatory, anti-rheumatic, anti-tumor, anti-phlegmatic, antiviral, astringent, aromatic, blood purifier, clear skin color, remove wound maggots, hepatic protective, stop liver obstruction, heals wound, stimulant and sedative in the food industries, as a coloring agent as well as an additive to impart flavor in curries (Ahmad *et al.,* 2010).

Chandarana *et al*. stated that turmeric is active against *B. subtilus*, *S. aureus* and *E. coli* due to the phenolic compounds present in turmeric like curcuminoids. The essential oil, alkaloid, curcumins, turmerol and veleric acid are accountable for antimicrobial activity of turmeric (Chandarana *et al*., 2005).

2.4.1.1.1. Mode of Antimicrobial Action of Curcumin

The mechanism of behind the antimicrobial action of different spices include the hydrogen bonding and hydrophobic interaction of various phenolic compounds to the membrane proteins, which cause cell membrane disturbance, disruption of cell wall and damage of electron transport chain. The antibacterial potential of aqueous extracts is possibly due to the anionic constituents like nitrate, chlorides, sulphates and thiocyanate in addition to several other compounds that are present naturally in plants. The ethanolic extracts exhibited better effects as dissolves organic compounds quickly resulting in release of larger amount of vigorous antimicrobial constituents (Juglal *et al*. 2002).

The thick structural components of gram-positive bacteria in this case can be accountable for the more interaction between curcumin, active components and the structural lipoproteins. The increased collaboration may outcomes in the inhibition of the gram-positive bacteria (Darout *et al*., 2000).

2.4.1.1.2. Synergism of Curcumin with Antibiotics against *S. aureus*

S. aureus In addition to showing potent antibacterial activity when used alone, curcumin also exerts marked activity against *S. aureus* when used at sub inhibitory dose in combination with various other antibiotics

(Keen *et al.,* 2010 ; Taneja *et al.,* 2013). These findings are interesting since curcumin is naturally derived from turmeric, which is one of the major ingredients of Asian cuisine ,of note, crude turmeric extracts have previously shown marked antibacterial activities against *S. aureus (*Stefani *et al.*, 2012).

Curcumin inhibits the growth of both Gram-positive and Gram-negative bacteria (Tyagi *et al.,* 2015). *S. aureus* is one of the Gram-positive strains that is susceptible to curcumin-mediated inhibition. *S. aureus* is a pathogen that causes various infections including infective endocarditis (IE), bacteremia, skin and soft tissue, osteoarticular, and pleuropulmonary infections (Tong, *et al.,* 2015).

Over the years, *S. aureus* has evolved and developed multiple strategies to evade human immune system and to resist antibiotics treatment. This has given rise to the evolution of MRSA, and the emergence of healthcare associated (HA) and community-associated (CA). MRSA has caused amajor problem to the human society (Miller and Kaplan 2009).

The curcumin may have acted on the target or pathway related to the development of drug resistance, hence restoring the killing effect of the drugs (Klingenberg *et al.,* 2004) .This may be one of the mechanisms on how curcumin enhances the effect of antibacterial drugs, especially when they are targeting *S. aureus*-infected human cells.

2.4.1.2. Berberine

Berberine (BER) is an isoquinoline quaternary alkaloid (5,6-dihydrodibenzo quinolizinium derivative) isolated from many medicinal plants, such as *Hydrastis canadensis, Berberis aristata, Coptis chinensis, Coptis rhizome, Coptis japonica, Phellodendron amurense*, and *Phellodendron chinense schneid* (Xia *et al.*, 2022) . BER is used to treat

diarrhea and bowel disorders in China, with the potential to be used for the treatment of many diseases (Tillhon *et al.*, 2012)

Furthermore, BER has received extensive attention or its role as an antimicrobial (Roser *et al.*, 2016) , antifungal,(Xiao *et al.*, 2015) anti-tumor,(Gu *et al.*, 2020) and anti-diabetes drug (Zhang *et al.*, 2020) However, the anti-MRSA mechanism of BER is still unclear.

Antibiotics play a crucial role in ensuring the safe supply of food-producing animal in sustainable animal production systems (Hassan *et al.*, 2019 ; Chen *et al.*, 2020). However, extensive and uncontrolled exposure of various antibiotics has caused an unprecedented risk from bacterial resistance (Troeman *et al.*, 2019 ; Van Boeckel *et al.*, 2020). In particular, *S. aureus* has shown resistance to most currently approved antibiotics, such as penicillin and tetracycline (Lin *et al.*, 2020 ; Hobson *et al.*, 2021).

Therefore, it is imperative to exploit new effective and innovative antibacterial agents to defend the invasion caused by drug-resistant *S. aureus* strains, natural products were significant resources of alternative classes of antibiotics with new antibacterial scaffolds (Porras *et al.*, 2020).

Berberine, a natural isoquinoline alkaloid isolated from several medicinal herbs such as *Berberis vulgaris* and *Rhizoma coptidis*, has been used as a nonprescription medicine to treat infectious diseases (such as bacillary dysentery, acute gastroenteritis, and cholera) in China for 2000 years (Li *et al.*, 2019 ; Xu *et al.*, 2020).

2.5. Cell Viability

Cell viability is often defined as the number of healthy cells in a sample. Cell viability methods can be categorized into those which analyze whole population and which involve individual cells ((Louis and Siegel, 2011). The first, and probably best known, metabolic dye is3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide(MTT)). This assay relies on the conversion of soluble tetrazolium into insoluble purple formazan crystals by reduction. Other similar dyes have become increasingly popular, such as XTT (sodium3¢-[1-(phenylaminocarbonyl)-3,4-(tetrazolium)-bis-(4-methoxy-6-nitro)- benzene sulphonic acid hydrate),MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), and WST derivatives. XTT and MTS are used in the presence of phenazinemetho-sulphate (PMS) which increases their sensitivity (Stoddart, 2011).

2.6. Cytotoxicity

Cytotoxicity is a complex process affecting multiple parameters and pathways. These parameters are cellular organelles that undergo changes in their activity as a result of apoptosis and necrosis induced by drug. Nuclear morphology, cell permeability and mitochondrial function, resulting in the loss of mitochondrial membrane potential and release of cytochrome C from mitochondria are good examples for cytotoxicity parameters (Sang *et al*., 2002).

Mitochondria considered as a cellular target for anticancer drugs. The Mitochondrial therapeutic approach against cancer uses strategies that either modulate the action of Bcl-2 family members at the mitochondrial outer membrane or induce the formation of the mitochondrial permeability transition (Borner, 2003).

Some anticancer drugs cause apoptosis by inducing mitochondrial membrane permeability changes that lead to the loss of mitochondrial membrane potential, decreased Bcl-2 levels, cytochrome C release, caspase-3 activation and formation of reactive oxygen species (Sang *et al*., 2002).

Topoisomerase I is essential for DNA replication, some anticancer drugs stabilize the DNA topoisomerase I cleavable complex and cause accumulation of single- stranded breaks in DNA, the collision of a DNA, Replication fork with the cleaved strand of DNA causes an irreversible double-strand DNA break, ultimately leading to cell death (Krzysztof *et al*., 2009).

Topoisomerase I induces an apoptotic cell death in cancer cells by inducing caspase activation such as caspase-3, -8, and -9 (Stiborova *et al*., 2012). Hydrogen peroxide may have an important function in cancer development. However, there is also compelling evidence that have shown that increasing the cellular levels of H_2O_2 may be an efficient way of killing cancer cells. That H_2O_2 can both produce apoptosis resistance and efficient inductor of apoptosis in cancer cells (Singh *et al*., 2007).

2.6.1. Cytotoxic activity of extracts of two plants against carcinogenic and non-tumorigenic cell line

Cancer is one of the most life-threatening diseases and possesses many health hazards in both developed and developing characterized by irregular proliferation of cells. The toxicity of chemotherapeutic drugs sometimes creates a significant problem in the treatment of cancer using allopathy or established medicine. Plants still have enormous potential to provide newer drugs and as such are a reservoir of natural chemicals that may provide chemoprotective potential against cancer (Izevbigie, 2003).

Recently, various therapies have been propounded for the treatment of cancer, many of which use plant-derived products. The medicines always played an important role in the global health. The health medicinal plants providing a new area of drug research (Desai *et al.*, 2008). The demand for plant-based medicines, food supplement, health products, pharmaceuticals, and cosmetics are increasing in both developing and developed countries due to the growing recognition that then a natural products are non-toxic, have less side effects and easily available (Verma *et al.*, 2014).

Secondary metabolites have been developed in nature form of different plant species, insects, fungi, algae, and prokaryotes during evolution in enormous diversity. Plant secondary metabolites can be defined as the compounds that play an important role in the interaction of the plant with its environment, but have no such role in maintaining the fundamental life processes in plants (Oksman , 2004).

Breast cancer is the most common invasive female cancer worldwide (Saonere, 2016 ; Ewaid and Al-Azzawi 2017). To find out drugs of natural origin without side effects and to find easily available and economically affordable therapies that can decrease the cost of treatment on the patient, the herbal medicines is the option, which can be used for the treatment of some cancers without or with less side effect. (Saonere, 2016), as well as it offer high compatibility with the physiology of the body by selectivity inhibiting of cancer cells proliferation, increasing cytotoxicity, and decreasing of cell differentiation. (Motamedi *et al.*, 2016) .

One of the basic limitations of most chemotherapies is the side effects induced by severe toxicity (Chatterjee *et al.*, 2011) .The main objective of any cancer treatment is removing away all of the malignant cells and leaving the normal cells in intact form as possible (Zaman , 2010) .

Skin cancer is one of the most common malignancies in dermatology. With the rapid development of industrialization and changes in people's living environment, the incidence of skin cancer has been increasing year by year, which has gradually developed into a worldwide public health problem (Gordon and Rowell 2015). Early specificity of diagnosis and treatment is still lacking; the current common treatment methods are surgery, radiation/chemotherapy, laser, and cryotherapy (Guy *et. al*, 2015).

Curcumin inhibits the proliferation of various tumor cells in culture and prevents carcinogen-induced cancers in rodents. In xenotransplant or sorthotransplant animal models, the growth of human tumors was inhibited by curcumin alone or in combination with chemotherapeutic agents or radiation (Kunnumakkara *et al.*, 2008).

Banerjee *et al.* 2010 reported that curcumin induced G2/M arrest and apoptosis, inhibited cell proliferation by inhibiting the assembly dynamics of microtubules, and further activated the mitotic checkpoint in MCF-7 cells. Furthermore, cells were accumulated in the G*(1)* phase of the cell cycle, and curcumin suppressed the expression of zeste homolog 2 (*EZH2*) gene via the stimulation of three major members of the mitogen-activated protein kinase (MAPK) pathway: c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase (Hua *et al.*, 2010).

Berberine (BER) is an isoquinoline alkaloid commonly extracted from a medicinal herb, Rhizoma coptidis, and plays a significant role in the inhibition of pathogenesis via suppression of intracellular reactive oxygen species (ROS) generation. This alkaloid stimulates the pro-inflammatory responses, through constraining various signaling pathways linked with inflammation (Olcum *et al.*, 2020 ; Bagherniya *et al.*, 2021), and is frequently used for intestinal infections (Tang *et al.*, 2009).

It as anti-inflammatory and antimicrobial properties, anti-diabetic and antioxidant effects and multiple pharmacological properties. BER has further been shown to have antitumor effects on many cancer cell lines, including leucocytes, liver, lung, stomach, colon, skin, oral, esophageal, brain, bone, breast, and genital cancer cells (Guamán *et al.*, 2014).

3: Materials and Methods

3.1. Materials

3.1.1. Laboratory Equipments and Apparatus

The equipment used in this study with their sources are given in table (3-1).

Table (3-1): Equipment and instruments used in this study

Equipment	Company	Country
Autoclave	Hirayama	Japan
Biological safety cabinet	Labogene	Korea Denmark
Distillator	FineTech	Korea
E-graph –UV (Gel documentation)	ATTO	Korea
Electrophoresis power	supply Pelex	France
flasks and beakers	Hirschman	Germany
Gas burner	GFL	Germany
Gel electrophoresis	Cleaver	USA
Incubator	Memmert	Germany
Loop, wood sticks	Roche	China
Medical injection Syringes	MEDECO	UAE
Micro centrifuge	Himedia Beckman	India Germany
Micropipettes	Slamid	USA
Microcentrifuge tubes	BIO BASIC	Canada
Medical cotton	Kardelen	Turkey
Nanodrop Instrument	Bioneer	Korean
PCR system/Conventional	Cleaver	USA
Refrigerator	Concord	Lebanon
Spectrophotometer	Optima sp3000	Japan
Sensitive electron balance	A & D	Japan
Swab Transport media	Oxoid	(England)
Vortex	Gemmy	Taiwan
Water bath	GFL	Germany

3.1.2. Chemicals:

Table (3-2): Chemicals and biological materials used in this study with their origins

Chemicals and Biological materials	Company/country
Agarose	Froggabio, Canada
Nuclease-Free-Water	Bio basic, Canada
DNA Ladder Marker 100bp	Promega, USA
Ethanol 70%	BDH, UK
Primers	Macrogen, Korea
Tris borate TBE buffer (loading buffer)	Promega ,USA
PCR pre mix (master mix)	Bioneer, Korea
Master mix ARMS PCR – Kit	Promega, USA
DNA loading dye	Promega/USA
Red safe nucleic acid staining solution 1ml	BDH, England
Distilled water	
Ethedium Bromide	Promega/USA
Glycerol	BDH / England
Curcumin extract	Life Plants /SQUARED NUTRITION
Barbrein extract	Life Plants / SQUARED NUTRITION
EDTA, Dimethyl sulfoxide (DMSO), Trypsin, Fetal bovine Serum, RPMI- 1640 Media, Phosphate Buffer Saline, Sodium bicarbonate	Sigma / USA

3.1.3. Kits

Table (3-3) kits that used in this study with their origins

Subjects	Company / country
VITEK-2 compact system kit	Biomerieux/ France
G-spinTM Genomic DNA Extraction Kit	iNtRON Biotechnology/ Korea
Go Taq® Green Master Mix Kit	Promega /USA
GoTaq® 1-Step RT-qPCR System	Promega /USA
MTT solution 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl -2H- tetrazolium bromide	Intron /Korea
Solubilization solution	Intron Korea

3.1.4. Antibiotic discs

Table (3-4): Antibiotic discs used in this study:

Antibiotic Disk	Potency	Company /Origin
Amikacin	10 µg	Bioanalyase / Turkey
Gentamycin	10 µg	Bioanalyase / Turkey
Tobramycin	10 µg	Bioanalyase / Turkey
Kanamycin	30 µg	Bioanalyase / Turkey
Streptomycin	25 µg	Bioanalyase / Turkey
Neomycin	30 µg	Liofilchem / Italy

3.1.5. Culture Media

Table (3-5): Culture media used in this study

Medium	Company/ Origin
Brain-Heart-Infusion broth	Hi-media (India)
Nutrient agar	Hi-media (India)
Blood agar base	Hi-media (India)
Manitol salt agar	Hi-media (India)
Muller Hinton agar	Hi-media (India)
Roswell Park Memorial Institute – 1640 Medium	Gibco/ U.K
Serum-medium	Gibco/ U.K
Nutrient broth	Biolife (Italy)

3.1.6. Primer

Primers that used in this study were provided by Macrogen company from Korea as following table (3-6).

Table (3-6): The name, sequence and product size of primers

Target gene	Primer sequence	Size	Reference
aac(6') /aph(2'')	5'CAGAGCCTTGGGAAGATGAAG-3' 5'-CCTCGTGTAATTCATGTTCTGGC-3'	348 bp	Khosravi *et al.*, 2017
aph(3)-IIIa	5'-GGCTAAAATGAGAATATCACCGG-3' 5'-CTTTAAAAAATCATACAGCTCGCG-3'	523 bp	
ant(4)-Ia	5'-CAAACTGCTAAATCGGTAGAAGCC-3' 5'-GGAAAGTTGACCAGACATTACGAA-3'	294 bp	
mecA	5'-CCT AGT AAA GCT CCG GAA-3' '5-CTA GTC CAT TCG GTC CA-3'	314 bp	Ardic *et al.*,2006
16s rRNA	5'-AGAGTTTGATCMTGGCTCAG-3' 5'-GCTGCCTCCCTAGGAGT-3'	355 bp	Patel, 2001

3.2. Methods

3.2.1 Study Design

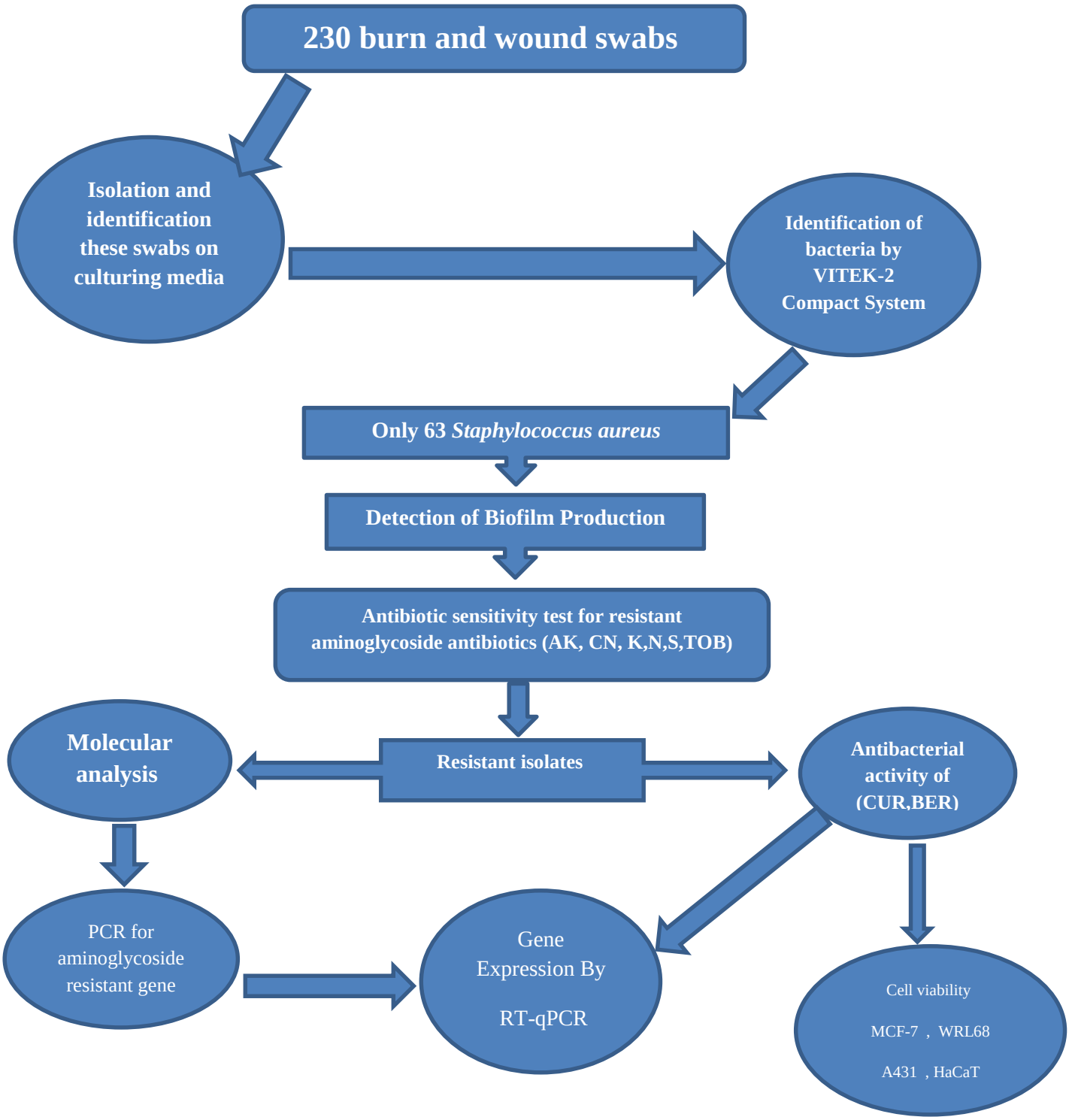


Figure (3-1) :- Study design

3.2.2. Sample collection and bacterial isolates

Study population included 230 participants with burn wound infection admitted burn unit in Imam AL-Sadiq Hospital, Al-Hilla teaching Hospital and Specialized Burn Center in Medical City of Baghdad and Kufa from February to September 2022. Burn wound swabs were taken from all patients registered in the study and immersed in Stuart's transport medium. Swabs were collected from infected wound following cleansing of any remnant ointment. After collection, all swabs were inoculated on Nutrient agar , Blood agar and Manitol salt agar and incubated at 37ºC for 24 h. for morphological examination of these swabs and colonial morphology, production of β hemolysis on blood agar and production of pigmentation on Manitol salt agar revealed 63 isolates of Staphylococcus that were confirmed to be *S. aureus* ultimately confirmed by the Vitek 2 Advanced Expert System (bioMerieux, Marcy l'Etoile, France).

3.2.3. Ethical Approval:

All samples of the present study were collected after obtaining ethical clearance from the Ethics Committees of Babylon health office and agreement of doctor management of burn unit in Imam AL-Sadiq Hospital, Specialized Burn Center in Medical City of Baghdad and Kufa.

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number M220109 in 17-1-2022 to get this approval .

Moreover, this study was approved by the ethical research committee in the College of Science, Babylon University, and Babylon health directorate**.**

3.2.4. Laboratory prepared culture media

All media (Nutrient, Manitol and Muller Hinton agar) were prepared according to the manufacturing company instructions .The constituents were dissolved in distilled water completely, sterilized by autoclaving at 121ºC for 15min at 15 pound/inch2. After cooling to 45-50 °C each medium was dispensed into sterile petri dishes in case of agar media and in sterilized screw tubes in the case of broth media. The work performed in laminar flow cabinets. Then, poured media were incubated for 24hr at 37 C° to ensure sterility. (MacFaddin , 2000).

3.2.4.1. Blood agar medium

This medium was prepared according to the instruction of manufacturer company, sterilized by autoclaving at 121ºC for 15min. after cooled to 50ºC, 5ml of sterile human blood was added for each 100ml of the medium, mixing well then poured in a sterile Petri-dish. This medium is suitable for the isolation and cultivation of bacteria and for the detection of haemolytic activity and the kind of haemolysis (Atlas, 2005).

3.2.4.2. Mueller-Hinton Plates

Mueller-Hinton agar was prepared according to the manufacturer's instructions then the medium was cooled to 45-50 ºC and poured into the plates, allowed to set on a level surface to a depth of approximately 4mm. When the agar was solidified, the plates were stored at 4 ºC until use.

3.2.5. Preservation of bacterial isolates

3.2.5.1. Short time preservation

A single pure colony of bacterial isolate was streaked on the nutrient agar culture plates and on the nutrient agar slants; incubated at 37ºC for 24 hr, sealed well and stored at 4ºC in the refrigerator one month for the plates and three months for the slants(Harely and Prescott, 2002)

3.2.5.2. Long Time Preservation

Glycerol method

A nutrient broth was inoculated by a loop of overnight pure bacterial culture, and incubated at 37 ºC .After 18 hr, glycerol was added to the inocula in a final concentration of 15-30% and stored at (-20 C) for 12-18 months (Vandepitte *et al*,. 2003).

3.2.6. Identification of bacteria by VITEK-2 Compact System

Suspension of bacteria was prepared according to the manufacturer's instructions. An adequate number of colonies was obtained by transferring an overnight pure culture and suspending it in 3.0 ml of sterile saline in (polystyrene) test tube. Adjustment of turbidity to 0.5 McFarland was made. Employing a Densi-Chek turbidity meter. Finally, the vitek-2 chamber with the specimen suspension tubes was loaded with the Gram negative -ID, and Gram positive -ID cassette (Karagöz *et al*., 2015).

3.2.7. Detection of Biofilm Production

Biofilm production by isolated burn wound swabs in this study was detected by phenotypic method which included Tissue Culture Plate

Method “TCPM “ . Biofilm production was graded into strong, moderate and non/weak. Strong and moderate results were interpreted as positive biofilm production, while, non/weak results were interpreted as negative biofilm production.

3.2.7.1. Tissue Culture Plate Method (TCP)

TCP method was the gold standard test for detection of biofilm formation (Aparna and Yadav 2008 ; Hassan *et al.*, 2011).

1. A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose.
2. The inoculated broth was then kept in the incubator at 37°C for 24 h.
3. Bacterial suspensions were further diluted 1:100 with fresh medium.
4. Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells, were filled by 200 µl of the prepared bacterial suspension.
5. Similarly, control organisms were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and toidentify non-specific binding. After incubation at 37 °C for 24 h.
6. The plate was gently tapped to remove the content of the wells followed by washing with 200 µl of phosphate buffer saline.
7. The washing step was repeated four times to remove any free bacteria present in the wells.
8. Sodium acetate (2%) were added to the wells and kept for 30 min. in order to fix the biofilms formed by bacteria attached to the wells.
9. Staining of the fixed biofilms was conducted using crystal violet (0.1%).

10. After 30 min., the wells were thoroughly washed by deionized water to remove any extra stain.
11. After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms.
12. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation.
13. The OD values were calculated and biofilm production was graded into strong, moderate and non/weak (Table 4-4) as described in previous studies (Panda *et al.,* 2016 ; Sultan and Nabiel 2019).

3.2.8. McFarland test

3.2.8.1. Preparation of McFarland standard solution

McFarland's standard solution 0.5; it is the turbidity standard solution which is the most widely used method of inoculums preparation or standardization. This solution has specific optical density to provide turbidity comparable to that of bacterial suspension containing 1.5×10^8 CFU/ml. This solution was prepared as the follows: Collee *et al*., 1996; Benson,2001)

Solution (A): 1g of barium chloride (BaCl2) was dissolved in 100ml of distilled water.

Solution (B): 1ml of concentrated sulphuric acid (H_2SO_4) was added and the volume was completed to 100ml by distilled water.

0.5ml of solution (A) was added to 99.5ml of solution (B) and stored in a dark bottle until used.

3.2.8.2. Inoculate Preparation (Turbidity Standard)

To prepare the inoculate, colonies from overnight culture of staphylococcal isolates were transferred to 5 ml tube of normal saline to obtain culture with 1.5×10^8 CFU/ml by adjusting to 0.5 McFarland standard.

3.2.9. Susceptibility Test of Antimicrobial agents

Drug susceptibility testing was performed by disk diffusion method according to CLSI guidelines (Weinstein and Lewis 2020). The standard bacterial suspension of *S. aureus* with turbidity equal to 0.5 McFarland was inoculated on Mueller Hinton agar (Merck, Darmstadt, Germany), using the following aminoglycoside antibiotics: gentamicin (10 µg), amikacin (10 µg), kanamycin (30 µg), tobramycin (10 µg), streptomycin (25 µg) and neomycin (30 µg) [Mast Co., UK]. The plates were incubated at 37 'C for 18-24 h. and the diameter of zone of growth inhibition were measured and compared with standard values.

3.2.9.1. Modified Kirby-Bauer method

This test performed as the following:-

1- From an overnight culture plate, 4-5 colonies of bacterial isolate were picked up by sterilized inoculating loop and emulsified in 5ml of sterile normal saline until the turbidity is approximately equivalent to that of the McFarland No. 0.5 turbidity standard.

2- A sterile swab was dipped into the bacterial suspension, any excess fluid was expressed against the side of the tube.

3- The surface of a Mueller-Hinton agar plate was inoculated by bacterial isolate as follows: The whole surface of the plate was streaked with the swab, then the plate was rotated through a 45º angle and streaked the whole surface again; finally the plate was rotated another 90º and streaked once more.

4- By a sterile forceps the antimicrobial disc was picked up and placed on the surface of the inoculated plate. The disc was pressed gently into full contact with the agar. All plate content 3 disc.

5- The step (4) was repeated to all antimicrobial discs under the test, spaced evenly a way from each other.

6- The plates were incubated at 37ºC for 18-24 h.

3.2.9.2. Reading the Results

After incubation, the plates were examined for the presence of inhibition zone of bacterial growth (clear rings) around the antimicrobial discs, if there was no inhibition zone the organism was reported as resistant to the antimicrobial agent in that disc. If a zone of inhibition surrounded the disc, the diameter of the zone of inhibition was measured (by millimeters) and compared their sizes with values listed in a standard chart (Weinstein and Lewis 2020). Table (3-7).

Table (3-7) Diameter interpretive standards of inhibition zones according Weinstein and Lewis 2020.

Antibiotic disk	Potency	Resistant	Intermediate	Sensitive
Amikacin	10 µg	≤ 14	15 – 16	≥ 17
Gentamycin	10 µg	≤ 12	13 – 14	≥ 15
Tobramycin	10 µg	≤ 12	13 – 14	≥ 15
Kanamycin	30 µg	≤ 13	14 – 17	≥ 18
Streptomycin	25 µg	≤ 12	13 – 14	≥ 15
Neomycin	30 µg	≤ 12	13 – 14	≥ 15

3.2.10. Genotypic methods (Nucleic acids techniques):

3.2.10.1. Genomic DNA isolation from various samples

G-spinTM Genomic DNA Extraction Kit for Bacteria is ideal for purifying DNA from small amounts of starting materials.

3.2.10.2. Descriptions of G-spinTM Genomic DNA Extraction Kit

G-spinTM Genomic DNA Extraction Kit are designed for rapid isolation of genomic DNA from a variety of sample sources including fresh or frozen animal cells/tissues (for Cell/Tissue) and Gram-negative & Gram-positive bacteria (for Bacteria), yeast (for Yeast), or bloods (for Blood). The purified DNA is free of contaminants and impurities and is ideal for all PCR, Southern blotting, RAPD, and sequencing applications.

G-spin TM kit uses advanced silica-gel membrane technology for rapid and efficient purification of genomic DNA without organic extraction or ethanol precipitation. Furthermore, G-spin TM buffer system is optimized to allow rapid and simple cell lysis followed by selective binding of DNA to the column. By using procedure purify DNA from a variety of target source can be obtained within 20-40 min.

3.2.10.2.1. Protocol (For Gram-Positive Bacteria)

1. Harvest 1-2 ml of cells (OD_{600}: 0.8-1.0) by centrifuging at 13,000 rpm for 1 min.Remove supernatant.

2. 50 µl was added of Pre-Buffer and 3 µl of Lysozyme solution, mix well.

3. Incubated at 37°C for at least 15 min.

4. 250 µl was added of G-Buffer solution, and invert-mix well.

5. Incubate at 65°C for 15min.

6. 250 µl was added of Binding Buffer, and completely mix well by pipetting (at least 10 times) or gently vortexing.

Note: This step conduces to pass efficiently cell lysates through a column and increase gDNA binding onto column resins and important for efficient deproteinization.

7. Cell lysates loading on column and centrifuge at 13,000 rpm for 1 min.

Note : The maximum volume of the column reservoirs 800 µl. For sample volumes of more then 800 µl, sample load and spin again.

8. To wash, 500 µl of Washing Buffer A was added to column and centrifuge for 1 min at 13,000 rpm.

9. Solution removed. 500 µl of Washing Buffer B was added to column and centrifuge for 1 min at 13,000 rpm.

10. Solution removed and centrifuged for 1 min at 13,000 rpm.
11. Place the G-spin TM column in a clean 1.5 ml microcentrifuge tube (not provided), and added 50-200 µl of Elution Buffer directly onto the membrane.
12. Incubated at RT for 1 min, and then centrifuge for 1 min at 13,000 rpm.

3.2.10.2.2. Estimation of DNA Concentration and Purity

The DNA concentration of samples were estimated using Nanodrop by putting 1µl of the extracted DNA in the instrument to detect concentration in ng/ µm and purity detected by noticing the ratio of O.D. 260/280 to detect the concentration of DNA samples with protien , the accepted 260/280 ratio for pure DNA is between(1.7- 2) DNA quality could be assessed by 1% agaros gel electrophoresis (Russell and Sambrook 2001).

3.2.11. Detection of Aminoglycoside Genes by Polymerase Chain Reaction

The detection of *S. aureus* specific species gene, as well as the genes encoding the resistance for aminoglycosides antibiotic, was carried out by the amplification of specific sequences within the target gen using the polymerase chain reaction technique. The experiment was carried out using a mixture of specific sets of primers designated for each target gene that were mixed with the DNA sample (template) and a master mix reagent which contains (*Taq* polymerase, PCR buffer, MgCl2 and dNTPs).The final constituent was the deionized water. The

reaction mixture was mixed and centrifuged for 3 seconds to collect the drops from walls in order to ensure the final volume of 25µl, and then transferred to a thermal cycler to start reaction according to the steps of the specific program.

3.2.11.1. Primers Preparation

The primers were supplied by the Applied Biosystems Company as a lyophilized product of different Picomole concentrations. The Applied Biosystems company protocol was adopted for primer resuspension, by bringing the final concentration of primers to 10 pmol/µl of TE buffer, and stored at -20 ºC until being used.

3.2.11.2. Working Solution

PCR Pre Mix was accomplished after several trials. Thus the following mixture was adopted table (3-8) .

Table (3-8) The Master Mix components of *mecA, aac(6')/aph(2''), aph(3)-IIIa* and *ant(4)-Ia* gene PCR

Component	Concentration	Total amount
Nuclease Free Water	------	4.5 µl
Green Master Mix (2X) (Promega)	1X	12.5 µl
F Primer :1	0.1- 1.0 µM	2.5 µl
R Primer :2	0.1- 1.0 µM	2.5 µl
DNA sample	<250 ng	3 µl
Total Volume	---------	25 µl

3.2.11.3. PCR Program

To detect the mecA gene amplification, the PCR program was adopted table (3-9).

Table (3-9) : The PCR program for the *mecA* gene amplification

No.	Step	Temperature	Time	No. of cycle
1.	Initial denaturation	95ºC	3 min	1
2.	Denaturation	95 ºC	30 sec	35
3.	Annealing	54 ºC	30 sec	
4.	Extension	72 ºC	30 sec	
5.	Final extension	72 ºC	5 min	1

Table (3-10) : The PCR program for the , *aac(6')/aph(2'')*, *aph(3)-IIIa* and *ant(4)-Ia* gene amplification.

No.	Step	Temperature	Time	No. of cycle
1.	Initial denaturation	95ºC	3 min	1
2.	Denaturation	95 ºC	30 sec	35
3.	Annealing	55 ºC	40 sec	
4.	Extension	72 ºC	40 sec	
5.	Final extension	72 ºC	5 min	1

The PCR products were examined by electrophoresis on 1.5% agarose gels at 85volt for one 70 min. and then photographed using using a digital camera in gel documentation device.

3.2.11.4. Protocol of Gel Electrophoresis (Sambrook *et al*., 1989)

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

3.2.11.4. 1. Preparation of agarose gel 1%:

1- Agarose powder (0.5 gm) was added to the buffer.
2- The amount of 1 X TBE (50 ml) was taken in a beaker.
3- The solution was heated to a boiling point, using a water bath until all gel particles were dissolved.

4-Tow µl of ethidium bromide (5 mg/ml)) was added to the agarose solution.
5- The agarose was stirred in order to be mixed and to avoid bubbles.
6- The solution was left to cool down at 50 – 60^{o}C.

3.2.11.4.2. Casting of the Horizontal Agarose Gel

After fixing the comb in 1 cm away from one edge of the gel tray, the agarose solution was poured into the gel tray. The agarose was allowed to solidify at room temperature for 30 minutes. The fixed comb was carefully removed and the gel tray was placed in the gel tank. The tank was filled with1X TBE buffer until the buffer reached 3-5 mm over the surface of the gel.

3.2.11.4.3.DNA Loading and Electrophoresis

Samples were loaded carefully into the individual wells of the gel, and then electrical power was turned on at 85 V for 70 minute. The DNA was then moved from cathode (-) to anode (+) poles. The Ethidium Bromide

stained bands in the gel were visualized using a UV transiluminator at 350 nm, and then photographed**.**

3.2.12. Preparation of Plant Extracts

3.2.12.1. Antibacterial activity of the aqueous curcumin extract

In this study: curcumin supplement (1800 mg/ capsule),as a SQUARED NEUTRITION / USA , was collected from Baghdad, Iraq. Different concentrations of the curcumin were prepared in warm distilled water by using serial dilution method. Experiment was carried out Dissolve 2g of curcumin extract in 10 ml of warm distilled water in four dilutions (200, 100, 50 and 25 mg/ml). One hundred microliters of test isolates were spread onto the surface of Muller Hinton Agar plates using a glass spreader, and the wells were filled with 50 µL of aqueous curcumin extract. The test plates were incubated at 37°C for 24 h. distilled water was used as a negative control. The antibacterial activity of the aqueous plant extracts was evaluated by measuring the inhibition zone in millimeters (Jorgensen and Turnidge 2015).

3.2.12.2. Antibacterial activity of alcoholic extract of berberine

Berberine supplement (900 mg/ capsule),as a SQUARED NEUTRITION / USA ,was collected from Baghdad, Iraq. Different concentrations of the berberine were prepared in by using serial dilution method. Experiment was carried out by dissolve 1g of berberine extract in 2ml of 70% ethanol in four dilutions (500, 250, 125, 62.5 mg/ml). One hundred microliters of test isolates were spread onto the surface of Muller Hinton Agar plates using a glass spreader, and the wells were filled with 50 µL of berberine alcoholic extract. The test plates were incubated at 37°C for 24 h. 70% ethanol used as a negative control. The antibacterial

activity of the alcoholic plant extracts was evaluated by measuring the inhibition zone in millimeters (Jorgensen and Turnidge 2015).

3.2.12.3. Synergistic effect of curcumin and berberine with Aminoglycoside antibiotic

The antagonistic activity of antibiotic with curcumin and berberine extract combination were determined by the modified disc diffusion method according to the CLSI guidelines. The MHA plates were seeded with the above antibiotic disc impregnated with curcumin and berberine (200 mg /ml) along with plain antibiotic disc taking as positive control, the MHA plates were kept at 4C° for 1 h. to allow the proper diffusion, after that kept at 37 C° for 24 h. The zones of inhibition were measured by using a caliper micrometer against the back of the petri plates (Varak and Priya, 2019).

3.2.13. Cytotoxicity and cell viability of curcumin and berberine on Cancer Cell Line and normal cell line.

This in vitro method was performed to investigate the possible cytotoxic effect of curcumin isolated from *Curcuma longa* and berberine isolated from *Berberis aristata* extract on tumor cell lines and normal cell line , which are breast, skin cancer cell line (MCF-7) and (A431) respectively , and normal human hepatic, keratinocyte cell line (WRL68) and (HaCaT) respectively.

1. MCF-7 Cell Line

Michigan Cancer Foundation-7 (MCF-7) was derived from the pleural effusion from a 69 year old female suffering from a breast

adenocarcinoma (Soule, 1973). This cell line obtained from Center of Biotechnological Research in Malaysia.

2. WRL 68 Cell Line

The human hepatic cell line WRL 68 exhibits morphology similar to hepatocytes and hepatic primary cultures. Cells have been shown to secrete albumin and alpha-feto protein and express liver specific enzymes such as alanine amino transferase (Asita and Salehhuddin, 2013).

3. A431 cell line

The A431 cell line is a human epithelial carcinoma cell line that was established from a tumor biopsy taken from a 56-year-old female patient. The A431 cell line is commonly used in cancer research and has been extensively studied in the context of epidermal growth factor receptor (EGFR) signaling and anti-cancer drug development (Rheinwald and Beckett 1981).

4. HaCaT cell line

The HaCaT cell line is a spontaneously transformed aneuploid immortal keratinocyte cell line that was established from adult human skin. The HaCaT cell line is commonly used in dermatological research and has been extensively studied in the context of skin biology, wound healing, and carcinogenesis (Boukamp *et al.*, 1988).

3.2.13.1. Solutions and Media Used in Tissue Culture Technique

Solutions and media used for cell culture were prepared according to Freshney, (2015).

3.2.13.1.1. Solutions

3.2.13.1.1.1. Antibiotic solution

3.2.13.1.1.1.1. Streptomycin (1g/vial):

It was prepared by dissolving vial contents in 5 ml of sterile distilled water to prepare a stock solution (200,000µg/ml). The stock was stored at -18ºC. And 0.5 ml of it was added to 1 litter of culture media.

3.2.13.1.1.1.2. Benzyl Penicillin:

It was prepared by dissolving the contents of one vial which has 106 IU in 5ml of sterile distilled water to prepare a stock solution (200,000 IU/ml). The stock was stored at -18ºC. And 1 ml of it was added to 1 litter of culture media.

3.2.13.1.1.1.3. Sodium Bicarbonate Solution

The solution was prepared by dissolving 2.2 g of NaHCO3 in 1000ml distilled water.The solution was sterilized by autoclaving and kept at 4ºC until use.

3.2.13.1.1.1.4. Phosphate Buffer Saline (PBS)

This buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15 g NaH2PO4 and 0.2g Na2HPO4 in 900ml of distilled water, pH was adjusted to 7.2. The solution was sterilized by autoclaving and stored at 4ºC until use.

3.2.13.1.1.1.5. Trypsin Solution

It was prepared by dissolving 1 g of trypsin powder in 100ml PBS and sterilized by filtration using Millipore's filter (0.22µm). The solution was dispensed into 10ml aliquots and stored at -20ºC.

3.2.13.1.1.1.6. EDTA Solution

It was prepared by dissolving 1 g of ethylene-diamine-tetra acetic acid (EDTA) in 100ml of PBS and sterilized in autoclave for 10 minutes. The solution was dispensed in 10 ml aliquots and stored at 4ºC.

3.2.13.1.1.1.1.7. Trypsin-EDTA Solution

It was prepared by mixing 20ml of trypsin solution, 10 ml EDTA solution and 370 ml PBS. The mixture was stored at 4ºC.

3.2.13.1.2. Media

3.2.13.1.2.1. Roswell Park Memorial Institute – 1640 Medium (RPMI)

A ready to use package (100 ml) RPMI was used throughout this study. The medium was already supplied with 4-(2-hydroxyethyl)-1piperazine-ethane sulfonic acid (HEPES) and L-glutamine as illustrated by manufacturer.

The medium was completed by adding the following ingredients:

Penicillin G	103 IU
Streptomycin	0.001 g
Sodium Bicarbonate	1%
Fetal Bovine Serum	10 %

3.2.13.1.2.2. Serum Free Medium

Serum free medium is RPMI-1460 excluded from fetal calf serum.

3.2.13.2. Cell Line Maintenance (Freshney, 2015)

The MCF-7, A431, HaCaT and WRL 68 cells were suspended in complete RPMI- 1640 medium and allowed to propagate in culture flasks for 24 hrs. in a humidified atmosphere, supplemented with 5% CO2 at 37°C. When the cells reached up to 80% confluency, the growth medium was removed, and the adhesive cells were washed with PBS solution twice.

Two to three mL of trypsin-EDTA solution was added to the flask and the flask was turned over to cover the monolayer completely with gentle shaking. The flask was incubated at 37°C for 1-2 min. until the cells were detached from flask surface. The trypsin deactivated by adding complete RPMI-1640 medium followed by distributing cell suspension to other flasks containing fresh complete RPMI medium. Cultured flasks were incubated at 37°C in 5% atmospheric CO2 incubator. The required concentration of cells was obtained using trypan exclusion cell counting method by mixing 1 volume of cell suspension with 1 volume of trypan blue stain. After 3 min waiting, the cells were counted microscopically using hemocytometer and applying the formula:

Total Cell Count mL-1 = Cell count x Dilution Factor (Sample Volume) x 10^4

3.2.13.3. MTT Protocol

The cytotoxic effect of curcumin and berberine compounds extracts was performed by using MTT ready to use kit (Intron Biotech):

3.2.13.3.1. Kit contents:

- MTT solution 1 ml x 10 vials.
- Solubilization solution 50 ml x 2 bottle.

3.2.13.3.2. Protocol:

- Cells (1 x 10^6 cells mL-1) were grown in 96 flat well micro-titer plates, in a final volume of 200 μL complete culture medium per each well. The microplate was covered by sterilized parafilm and shacked gently.
- The plates were incubated at 37◦C, 5% CO2 for 24 h.

- After incubation, the medium was removed and two-fold serial dilutions of Curcumin and Berberine (12.5, 25, 50, 100, 200, 400 μg mL-1) were added to the wells.
- Triplicates were used per each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37◦C, 5% CO2 for selected exposure time (24 h.).
- After exposure, 10 μL of the MTT solution was added to each well. Plates were further incubated at 37◦C, 5% CO2 for 4 h.
- The media were carefully removed and 100 μL of solubilization solution was added per each well for 5 min.
- The absorbance was determined by using an ELISA reader at a wavelength of 575 nm. The data of optical density was subjected to statistical analysis to determine IC50 the concentration of compound required to cause 50% reduction in cell viability for each cell line.

3.2.14. Gene expression

3.2.14.1. Effect of curcumin and berberine plant extracts on gene expression of some virulence factor

The qRT-PCR was performed for quantification expression detection of biofilm formation genes that normalized by housekeeping (16SrRNA) gene in *staphylococcus aureus isolates*. The method was carried out according to (Shakerimoghaddam *et al*., 2017) .Three isolates which formed a strong biofilm were chosen for studying expression of the (*mecA, aac(6')/aph(2''), aph(3)-IIIa* and *ant(4)-Ia* and *16SrRNA* gene) in *staphylococcus aureus* bacteria .

For *S. aureus* the concentrations was used of curcumin (3 mg/ml) and of berberine (125 µg/ml) that reduced growth of bacteria . For gene expression studies, 5 ml of nutrient broth media was inoculated with tested bacteria and adjusted to (0.5 McFarland) . For each bacteria , three groups was made , the first group treated with curcumin named as C, second group treated with berberine and named B , the last group was the control which was bacterial growth without any treatment was named (no. of isolate with gene) .

3.2.14.2. RNA extraction

Total RNA was extracted from bacterial suspension by using (Total RNA Extraction Kit). TransZol lyses cells with guanidine isothiocyanate. In the process of sample lysis, TransZol can maintain the integrity of RNA .The contents of kit is demonstrate in table (3-11) .

Table (3-11) Basic component of TransZol up kit that used in study.

Component	ET101-01
TransZol Up	100 ml
RNA Extraction Agent	20 ml
RNA Dissolving Solution	15 ml

3.2.14.2.1. Procedure

1. Suspension cells:-

a. Transfered suspension cells to a micro centrifuge tube .centrifuged the sample at 8000Xg for 2 min at 2-8 ºC, discard the supernatant.

b. Added 1 ml of TransZol to 10^7 cells.

c. Pipetting up and down until no visible precipitates are present in lysate.

d. Incubated at room temperature for 5 min.

2. Added 0.2 ml of RNA Extraction Agent for per ml TransZol used. the tube was shaked vigorously by hand for 15 sec. . Incubated at room temperature for 3 min.

3. Centrifuged the sample at 10000 Xg for 15 min. at 2-8 ºC. The mixture separates into a lower pink organic phase, an interphase and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is around 60 % volume of TransZol reagent.

4. Transfered the colorless, upper phase containing the RNA to a fresh RNase – free tube. Added 0.5 ml of isopropanol for per ml TransZol used. Mix thoroughly by inverting tube. Incubate at room temperature for 10 min.

5. Centrifuged the sample at 10000 Xg for 10 min. at 2-8 ºC .Discard the supernatant. Colloidal precipitate can be seen at the wall and the bottom of the tube.

6. Added 1 ml of 75% ethanol (prepared with RNase-free water), vortexing vigorously (add at least 1 ml of 75%ethanol for 1 ml TransZol used).

7. Centrifuge the sample at 7500 Xg for 5 min at 2-8 ºC .

8. Discard the supernatant. Air-dry the RNA pellet (about 5 min.).

9. RNA pellet is dissolved in 50-100 µl of dissolving solution .

10. Incubate at 55-60 ºC for 10 min. For long term storage, store the purified RNA at -70 ºC.

3.2.14.3. Estimation and purity of total extracted RNA

The extracted total RNA was checked by using Nanodrop (Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer. USA) that measured RNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm) as following steps:

- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2microliter of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.

- The Nanodrop sampling arm was lowered and 1microliter RNA sample were measured.

3.2.14.4. Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using **(GoTaq® qPCR Master Mix)** based on The BRYT Green® Dye in the GoTaq® qPCR Master Mix has spectral properties similar to those of SYBR® Green I in Real-Time PCR system and the qPCR master mix for target genes and housekeeping gene was prepared as in table(3-12) .

Table (3-12) Component of GoTaq® qPCR Master Mix kit that used in study.

qPCR master mix	Volume
GoTaq® qPCR Master Mix, 2X	5 × 1ml
GoScript™ RT Mix for 1-Step RT-qPCR	225µl
CXR Reference Dye, 30µM	200µl
MgCl2, 25mM	750µ
Nuclease-Free Water	2 × 13ml

3.2.14.4.1. GoTaq® 1-Step RT-qPCR Protocol

The final reaction volume in this protocol is 20µl. The volumes given here may be scaled for larger or smaller reaction volumes.

1. Thew the GoTaq® qPCR Master Mix and Nuclease-Free Water. Do not thaw the GoTaq® qPCR Master Mix at elevated temperatures (i.e., above room temperature).

2. Vortex the GoTaq® qPCR Master Mix for 3–5 sec. to mix. Vortex at low speed to avoid aeration.

3. Determined the number of reactions to be set up, including negative control reactions. Added 1 or 2 reactions to this number to compensate for pipetting error. While this approach does require using a small amount of extra reagent, it ensures that you will have enough reaction mix for all samples.

4. Prepared the reaction mix (minus RNA template) by combining the GoTaq® qPCR Master Mix, GoScript™ RT Mix, PCR primers and

Nuclease-Free Water as described below. The RNA template is added in Step 6. Vortex briefly to mix.

5. The appropriate volume was added of reaction mix to each PCR tube or well of an optical-grade PCR plate.

6. Added the RNA template (or water for the no-template control reactions) to the appropriate wells of the reaction plate.

7. Sealed the tubes or optical plate, and centrifuge briefly to collect the contents of the wells at the bottom. Protected from extended light exposure or elevated temperatures. The samples are ready for thermal cycling.

3.2.14.4.2. Thermal Cycling

The cycling parameters below are offered as a guideline and may be modifed as necessary for optimal results.

Table (3-13) The RT- PCR program for gene expression

Step	Time	Temp.	Cycles
Reverse transcription	15 mint.	≥37°C	1
Reverse transcriptase inactivation and GoTaq® Polymerase activation	10 mint.	95°C	1
Denaturation	10 sec.	95°C	40
Annealing	30 sec.	60°C	
Extension	30 sec.	72°C	

The data results of qPCR for target and housekeeping gene were collected and the expression analysis (fold change) used analyzed by using (**The CT Method Using a Reference Gene**) that described by (Livak and chmittgen, 2001) as following equations:

Gene expression ration (Fold change) = Ratio (reference/target) = 2^CT(reference) – CT(target).

3.2.14.5. Calculating Gene Expression (Gene Fold).

There are two strategies for analyzing qPCR data: absolute and relative quantification. The absolute quantification identifies the input gene amount based on a standard curve which created by Livak and Schmittgen. In contrast, the relative quantification determines changes in gene expression relative to a reference genes sample which is accomplished by Pfaffl (Pfaffl, 2001).

Errors caused by standard dilutions when creating a standard curve can also be avoided. In addition, sometimes the relative gene amount between two treatment groups is of more interest than exact DNA/RNA molecular numbers. Therefore, the relative quantification is widely performed.

Gene expression or gene fold or RQ (Relative quantification) value were calculated by Pfaffl equation (Pfaffl, 2001):

$$\mathbf{RQ = 2^{-(\Delta\Delta CT)}}$$

The gene fold was calculated firstly by collecting CT (CT – cycle threshold) average value from real time PC device for each triplicated sample then ΔCT value was calculated for each sample as follows:

$$\mathbf{\Delta\ CT = CT\ (gene\ of\ interest) - CT\ (reference\ gene)}$$

ΔCT is the difference in CT values for the gene of interest and reference gene for a given sample. This is essential to normalize the gene of interest to a gene, which is not affected by experiment. Calculating ΔΔCT value is found as follows:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

After calculating ΔΔ CT for all samples, the final equation is taken to calculate the gene expression (fold change) as follows:

$$\textbf{Fold gene expression RQ} = 2^{-(\Delta\Delta CT)}$$

3.2.15. Statistical analysis

The data values are presented as the mean ± S.D. Differences in mean values were analyzed by the IBM SPSS Statistics version 27 software (International Business Machines Corp., Armonk, NY, USA). Values with a $P < 0.05$ were considered to indicate statistical significance (Daniel and Cross, 2018).

4. Results and Discussion

4.1. Bacterial isolation and identification

A total number of 230 clinical samples swabs were collected from different patients from various burns and wounds units in several hospitals that included (Hilla Teaching Hospital, Imam Al-Sadiq Hospital, Specialized Burn Center in Medical City of Baghdad and Kufa) from February to September 2022. Swabs were collected from infected wound following cleansing of any remnant ointment. *Staphylococci* were identified depending on the conventional cultural After collection, all swabs were inoculated on Blood agar and Manitol salt agar and incubated at 37^{o}C for 24 hours.

4.1.1. Colony Morphology:-

Morphological Examination of culture smear, colonial morphology, show Beta hemolytic activity on blood agar due to the production of Beta hemolysin by isolates, making a clear zone surrounding the isolates.

On a mannitol salt agar, which is considered a selective and differential growth medium that which is used for encouraging the growth of staphylococci and which inhibit others by containing a high concentration (~7.5%-10%) of NaCl (selective) and for distinguishing two groups of staphylococci, mannitol fermenters (*S. aureus*) and mannitol non-fermentors (other staphylococci) by containing mannitol sugar and phenol red as an indicator.(Leboffe and Pierce, 2010). 63 isolates gave a positive result and were identified as *S.aureus* . the morphology characteristics of S. aureus which grow on different media as in table (4-1)& figure (4-1)

Table (4-1):-Culture Characteristics of *S. aureus*

No.	Culture media	Morphology of colonies
1.	Blood agar	Small, round, smooth, golden yellow ,raised, glistening ,hemolysis
2.	Mannitol salts agar	Yellow colonies

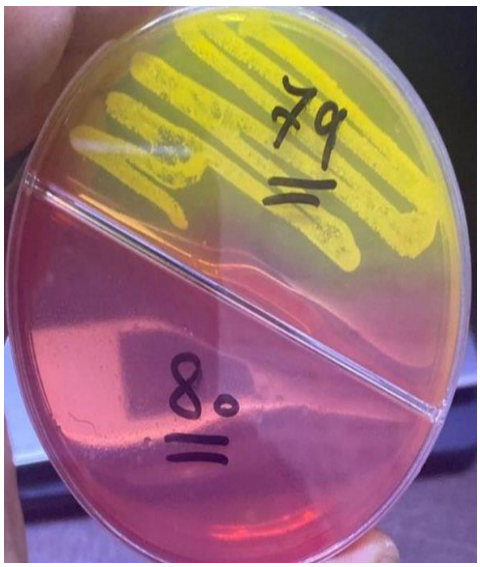
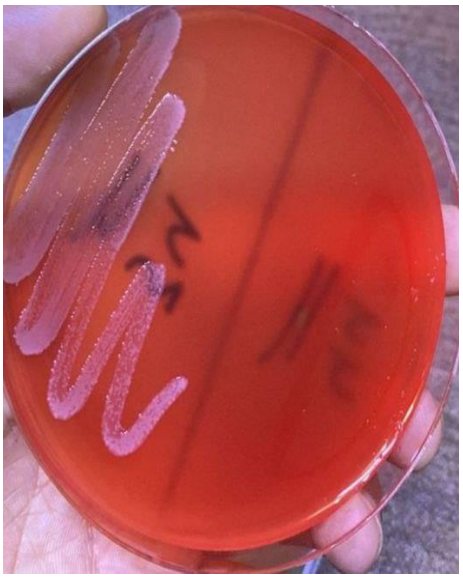
A.  **B.** 

Figure (4-1) : *S. aureus* isolates after 24 hours incubation.

A:- on Manitol salt agar **B:- on Blood agar**

Out of the 230 samples of burn and wounds patients, only 63 (27. 39%) isolates of *S. aureus* were isolated, the distribution was shown in Table (4-2).

Table (4-2) :- Distribution of *S. aureus* isolates

Sample sources	Sample collection locations			
	Hilla Teaching Hospital	Imam Sadiq Hospital	Specialized Burn Center \ Kufa	Specialized Burn Center \ Medical City – Baghdad
Burns No.= 50	-	10	26	14
Wounds No.= 13	13	-	-	-

Burn wound infections are one of the most important and potentially serious complications that occur in the acute period following injury. These wounds are subsequently colonized by microorganisms, including Gram-positive bacteria, Gram-negative bacteria and Yeasts, which derived from the host's normal flora (gastrointestinal flora, upper respiratory flora) and from the hospital environment (Peck , 2011).

Burn injury is the local response of a tissue, with or without systemic response, to an energy transfer form a physical (mechanical, thermal, electrical, radiation) or chemical source (Leboffe and Pierce 2010). Infection is an important cause of morbidity and mortality in hospitalized burn patients, in patients with burn over more than 40% of the total body surface area, 75% of all deaths following thermal injuries are related to infections.

The rate of nosocomial infections is higher in burn patients due to various factors like nature of burn injury itself, immunocompromised status of the patient, age of the patient, extent of injury, and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, colonization of the burn wound site, systemic dissemination of the colonizing organisms (Koller, 2014).

Previous studies such as Taneja N *et.,al* 2013 study have shown that there are several bacterial species which are able to readily infect burn wounds. *S. aureus* and *P. aeruginosa* have been found to be the most common species. Interestingly, the variation in bacterial flora and the colonization rate changes over time after the initial infection (Taneja *et al.,* 2013).

4.1.2. Bacterial isolates according to Vitek 2 Advanced Expert System

Among of total 230 swab isolates , 63 (27.39%) isolates *S. aureus* , 40 (17.39 %) *Staphylococcus lentus* were the predominant and the most common in Gram positive bacteria , while *P. aeruginosa* 127 (55.22 %) was predominant in Gram negative bacteria table (4-2).In VITEK2 Advanced Expert System system (bioMerieux, Marcy l'Etoile, France),the isolates were identified as *S. aureus* with probability of (98) % , *S. lentus* with probability of (92) % , *P. aeruginosa* with probability of (89) % , (table 4-3) Biochemical Characteristics of all bacterial isolates were done also by VITEK2 compact system , appendices (1) .These result are similar to (Taneja *et al.,* 2013) who found that *S. aureus* was the most common pathogens ,followed by *P. aeruginosa* .

Table (4-3): Identification of pathogenic bacteria depended on the colonial morphology and Vitek 2 system

Bacterial species	No. of isolates	Probability in Vitek 2 system
S. aureus	63	98 %
Staphylococcus lentus	40	93 %
Pseudomonas aeruginosa	127	93 %
TOTAL	230	-------

4.2. Detection of biofilm production among *S.aureus* isolates by Tissue Culture Plate Method

Tissue culture plate method detected biofilm formation of *S. aureus* for 63 isolates .It was found that among the isolates, 55 were biofilm producers and 8 were non-producing. the distribution of biofilm and non-biofilm producers based on the strength of biofilm formation were : weak, moderate, and strong, 40/63 were strong, 15/63 isolates were moderate and 8/63 isolates were weak/non-biofilm producers , with *P value* 0.000 .as shown in table (4-4) .

Table (4-4) Screening of *S. aureus* for biofilm formation by tissue culture plate (TCP) method.

Biofilm formation by TCP method		*P-value*
Results	**No. (%)**	
Strong	40 (63.5%)	0.000
Moderate	15 (23.8%)	
Weak/ non biofilm	8 (12.7%)	
Total	63 (100%)	

This data indicates that the TCP method is an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of staphylococci. Tissue culture plate method detected biofilm formation in 87.3 % of cases with significant statistics among the biofilm producers (*P-value* = 0.000). Similar study by Deotale *et al.,* 2015 revealed TCP method detected 81% bacterial isolates biofilm producer. Another study showed that 76% were bacterial biofilm producers detected by TCP method (Bin-Hameed 2021). Other study found that TCP detected 64% as bacterial biofilm producer (Sheriff *et al.,* 2016). While differences in the observations showed in other study that TCP detected 27% as bacterial biofilm producers (Ruchi *et al.*, 2015). Other study reported biofilm producer identified by TCP method 22% (Osungunna *et al.*, 2018).

4.3. Antibiotics susceptibility test:-

Lists of antibiotic susceptibility testing were created using documents and breakpoints from the Clinical Laboratory Standards Institute (CLSI , 2020) the European Committee on Antibacterial Susceptibility Testing (EUCAST) and the United States Food and Drug Administration (FDA).

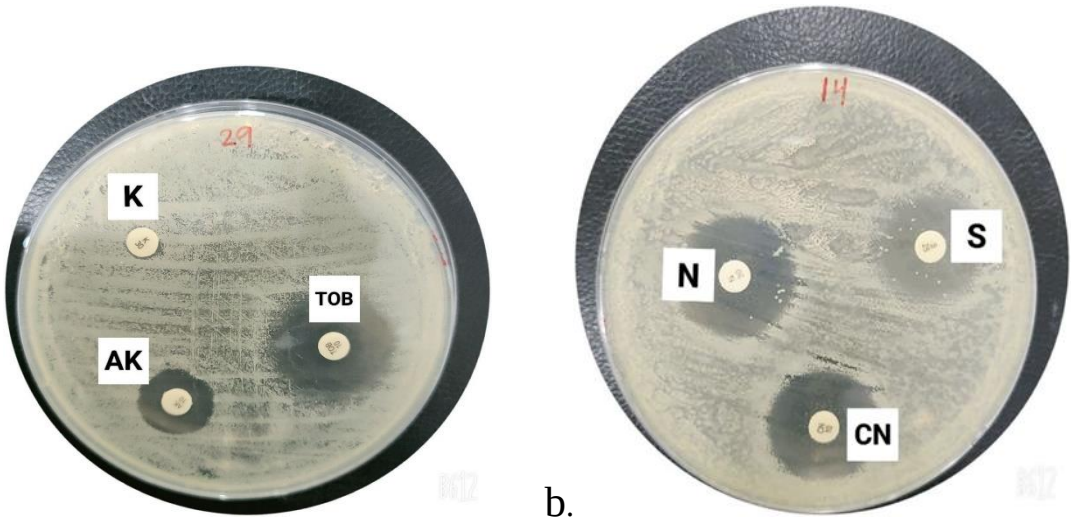
This study included six aminoglycosides antibiotics was determined by disc-diffusion method , which are Gentamicin (10 µg), Amikacin (10 µg), Kanamycin (30 µg), Tobramycin (10 µg), Streptomycin (25 µg) and Neomycin (30 µg) . The results was analyzing sensitivity pattern of antibiotics against 63 *S. aureus* isolates as in figure (4-2). The phenotypic prevalence of resistance to aminoglycoside antibiotics of *S. aureus* isolates to Amikacin were 30, Gentamycin 30, Tobramycin 36, Kanamycin 40,

Streptomycin 34 and Neomycin 34 isolates . Percentage resistance to theses antibiotics show in table (4-5).

Table (4-5) : Percentage Antibiotic Susceptibility Test for pathogenic bacteria

Antibiotics	**Antibiotic susceptibility of *S. aureus* (%)**		
	R	**I**	**S**
K (30 µg)	63.4	-	36.5
TOB (10 µg)	57.1	-	42.8
S (25 µg)	53.9	11.1	43.9
N (30 µg)	53.9	3.1	42.8
CN (10 µg)	47.6	4.7	47.6
AK (10 µg)	47.6	17.4	34.9

Key: (S) Sensitive, (I) Intermediate sensitive, (R) Resistant



a. b.

Figure (4-2):- Susceptibility test of *S. aureus* isolates to Aminoglycoside antibiotics after 18-24 h. incubation

a. For Amikacin, (AK) , Tobramycin(TOB) and Kanamycin (K).

b. For Streptomycin (S) , Neomycin (N) and Gentamycin (CN).

The emergence of antibiotics resistance in the majority of pathogenic bacterial strains is a cause of utmost concern in infectious bacterial diseases. Therefore, there is an inevitable need to identify the effective antibacterial agents which are more effective against microbial ailments with minimal side effects on host cells (Caruso and Poon , 2018).

According to the results of current study, determination of resistance to aminoglycoside antibiotics demonstrated that highest antibiotic resistance was found in Kanamycin, which included 40 isolates (63,4%) ,and then Tobramycin , which included 36 isolates (57,1%) , Also lowest antibiotic resistance was found in Amikacin and Gentamycin , which included 30 isolates (47,6%) this was consistent with the study of Shmitz *et al.* in Europe, the phenotypic prevalence of resistance to gentamicin and amikacin was reported in more than 30% of cases, which is which matches perfectly with the results of this study (Glad *et al.*, 2001) ,while it was not consistent with the study of Glad *et al*. in Kuwait , and Abdal *et al.*, 2014 . in Qom, and Ghotaslou *et al*., 2014 in Tabriz ,who demonstrated that highest antibiotic resistance was found in gentamicin, which included 59 isolates (52.21%). Several recent reports from Iran, demonstrated the increasing spread of MRSA strains and their high resistance to aminoglycosides (Emaneini *et al.*, 2009 ; Emaneini *et al.*, 2013**)**, and especially gentamicin resistance, represents a serious compromise in the treatment of Iranian patients as reported by (Fatholahzadeh *et al.*,2009).

Such differences in some parts of the reports in different regions can be attributed to changes in the strains based on the geographical region. To improve the effectiveness of these antibiotics, sometimes β-Lactam and aminoglycoside antibiotics are used in addition to aminoglycoside antibiotics ,aminoglycosides stop the translation mechanism of the

bacterium by binding to the 30S subunit of ribosomes and induce their antibacterial effects (Hu *et al*., 2015).Resistance to aminoglycosides is because of the presence of enzymes that cause changes in aminoglycoside antibiotics (Kelmani *et al.,* 2008) . These enzymes are coded by several genes including *aac* (6 ') *Ie / aph* (2 "), aph (*3')* - *IIIa1 and ant (4 ')* - Ia1 with the ability of intra-strain circulation by mobile genetic elements such as transposons . The three enzymes *aac(6′) / aph(2″), aph(3′)-III and ant(4′),* which are respectively coded by genes *aac (6 ') Ie / aph (2 "), aph (3')* - *IIIa1 and ant (4 ')* - *Ia1*, are among the most common modifying enzymes in various species of *S. aureus* and medically and clinically important (Abdal *et al.,* 2014).

Each of these enzymes code the resistance to a specific antibiotic; resistance to Gentamicin, Kanamycin and Tobramycin is due to the activity of *aac (6 ') Ie / aph (2 ")* enzyme, resistance to Neomycin, Tobramycin, Amikacin is due to the presence of *ant (4 ')* - *Ia1* gene and resistance to kanamycin and tobramycin is because of aph (3')-IIIa1 enzyme (Hamdad *et al.,* 2006 ; Shokravi *et al.,* 2015; Hu *et al.,* 2015). Additionally the resistance to more than one antibiotics may be attributed to efflux pumps (Al-Zuhairy and Al-Dahmoshi , 2020). The reasons for failure to treat resistant infectious strains include lack of using proper antibiotic disks for initial identification of strains resistant to aminoglycosides, low sensitivity of phenotypic methods, and lack of accurate and fast identification (Rahimi, 2013). One of the best and most sensitive ways for identification of resistant strains is detecting genes that distribute enzymes responsible for antibiotic resistance using polymerase chain reaction (PCR) technique.

4.4. Relationship of antibiotics resistance pattern with biofilm and non-biofilm producing *S. aureus* .

Bacteria in biofilm display dramatically increased resistance to antibiotics .In this study, analyzed the antibiotics resistance pattern of biofilm and non-biofilm producing of all isolates *S. aureus.* Biofilm forming isolates demonstrated increased resistance to the commonly used antibiotics compared to non-biofilm producers. *S. aureus* isolates biofilm producing in current study were found to be resistant to Amikacin, Gentamycin, Tobramycin , Kanamycin, Streptomycin and Neomycin 54.5 % , 58.2% , 72.7%, 80%, 60%, 63.6% respectively with significant statistical correlation of antibiotic resistance to Tobramycin, Kanamycin and Neomycin with *P-value* 0.003 , 0.0026, 0.0025 respectively as in table (4-6).

Table (4-6): **Antibiotics susceptibility test results of biofilm and non-biofilm producing for *S. aureus isolates***

	Biofilm producer			**Non-biofilm producer**			χ^2 test value	*P-value*
Antibiotic	55/ 63 (79.4%)			8/63 (20.6%)				
	R	**I**	**S**	**R**	**I**	**S**		
AK	**30**	**5**	**20**	**6**	**0**	**2**	**1.497***	**.0047**
CN	**32**	**5**	**18**	**5**	**1**	**2**	**1.239***	**.0088**
TOB	**40**	**0**	**15**	**5**	**1**	**2**	**6.991***	**.003**
K	**44**	**0**	**11**	**5**	**0**	**3**	**1.237***	**.0026**
S	**33**	**2**	**20**	**6**	**1**	**1**	**2.599***	**.0027**
N	**35**	**1**	**19**	**4**	**1**	**3**	**2.737***	**.0025**

S. aureus is known for its ability to form biofilms on various surfaces, which contributes to its persistence and the challenges in treating infections associated with this bacterium (Kebriaei, *et al.,* 2022).Some antibiotics are commonly used to treat *S.aureus* infections. Antibiotics can have reduced effectiveness against biofilms due to the limited penetration of the biofilm matrix and the presence of resistant subpopulations within the biofilm (Tuon, *et al.,* 2023). This pattern of resistance coincides with the study's findings of Davenport *et al.,* 2014 which reported biofilm producing *S. aureus* highly resistant to tobramycin.

The high resistance toward Tobramycin in current study was increased due to the excessive use of these drug for the treatment of both minor and more serious *staphylococcal* infections. Overall, the treatment of *S. aureus* biofilm-associated infections can be challenging due to the inherent resistance of biofilms. Addressing biofilm-related infections often requires a multifaceted approach, including the use of appropriate antibiotics, combination therapies, biofilm-disrupting agents, or approaches that enhance antibiotic penetration into the biofilm (Frieri, *et al,* 2017).

Results of the current study are coincides with Yang *et al.,* 2017 who found *S. aureus* biofilms incubated with kanamycin were significantly inhibited compared to the control ($P \leq 0.05$), and there results indicated that the different concentrations of kanamycin used are irregular in their ability to influence the inhibition of biofilm formation.

4.5. Molecular analysis

4.5.1. Detection Aminoglycoside genes in *S. aureus* isolates

Based on PCR test, only 50 *S. aureus* isolates were resistant to Aminoglycosides genes and mecA in primary screening, 12 isolates (24%) contained *mecA* gene. In addition, 32 isolates (64 %) had *aph(3')-IIIa1* gene and 6 isolates (12 %) had *aac(6')Ie/aph(2'')*, While the presence of the *ant(4')-Ia1* gene was not observed in any of the samples. as in table (4-7).

Table (4-7):- Genotyping distribution of resistance genes for *S. aureus* in burn wound of study

Isolates	**Aminoglycoside genes**				χ^2 **test value**	***P-value***
	aph(3)-IIIa	*aac(6')/aph(2'')*	*ant(4)-Ia*	***mecA***		
No .= 50	32 (64 %)	6 (12 %)	0	12 (24%)	61.760*	.000

* Significant count less than *P-value* 0.05.

Figure (4-3), (4-4) and (4-5) show the gel electrophoresis of these genes in *S.aureus* isolates.

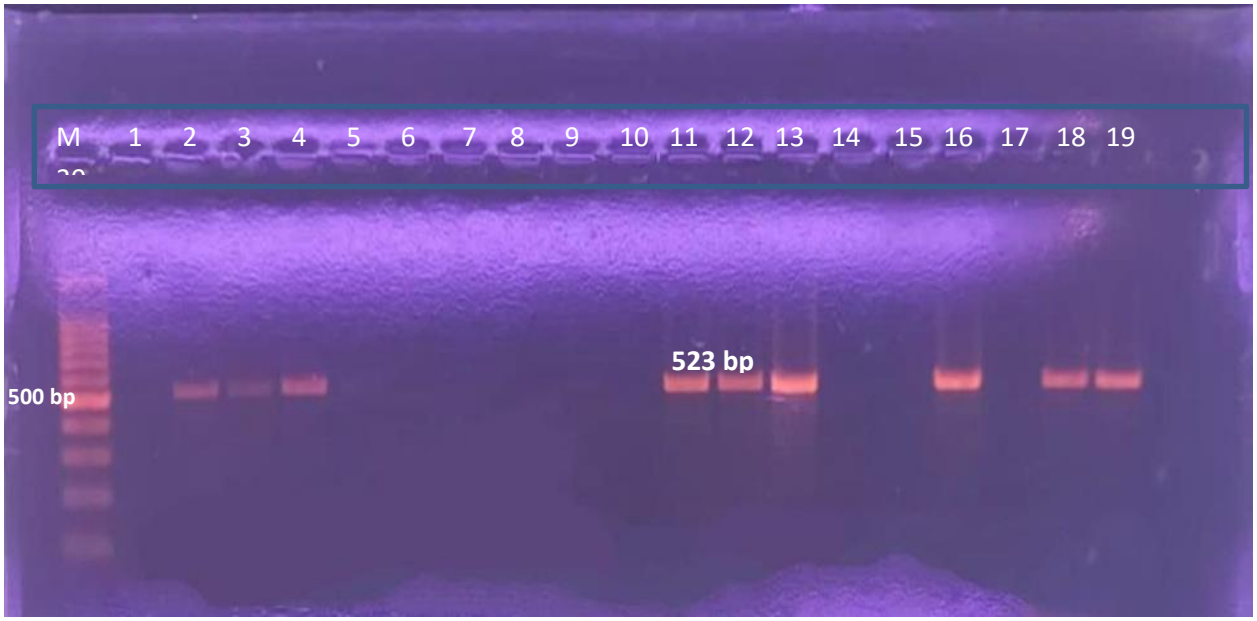
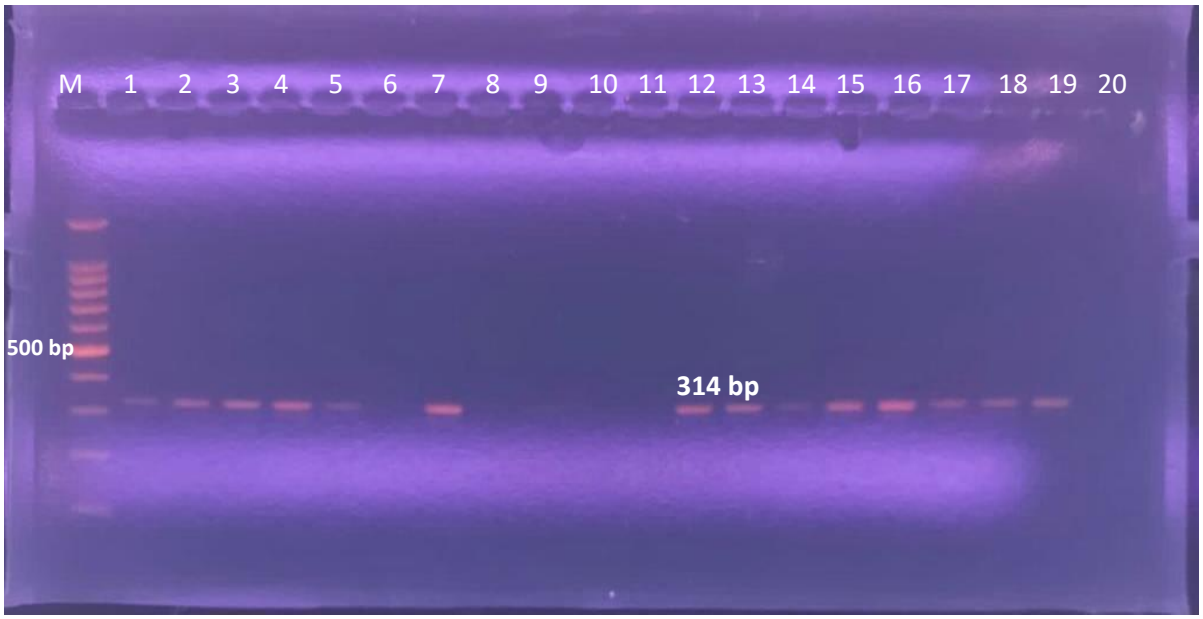


Figure (4-3):- Gel electrophoresis of *aph(3)-IIIa* gene for *S. aureus* bacteria in voltage (85V) time (70 minute)and 5µL of PCR product loaded in well (1-20). Lane M: DNA ladder (100bp). wells (2,3,4,11,12,13,16,18,19) are positive to presence this gene while (1,5,6,7,8,9,10,14,15,17,20) are negative to this gene .



Fifure (4-4):- Gel electrophoresis of *mecA* gene for *S. aureus* bacteria in voltage (85V) time (70 minute)and 5µL of PCR product loaded in well (1-20). Lane M : DNA ladder (100bp). All these sample are positive for the presence of this gene except well (6,8,9,10,11,20) are negative

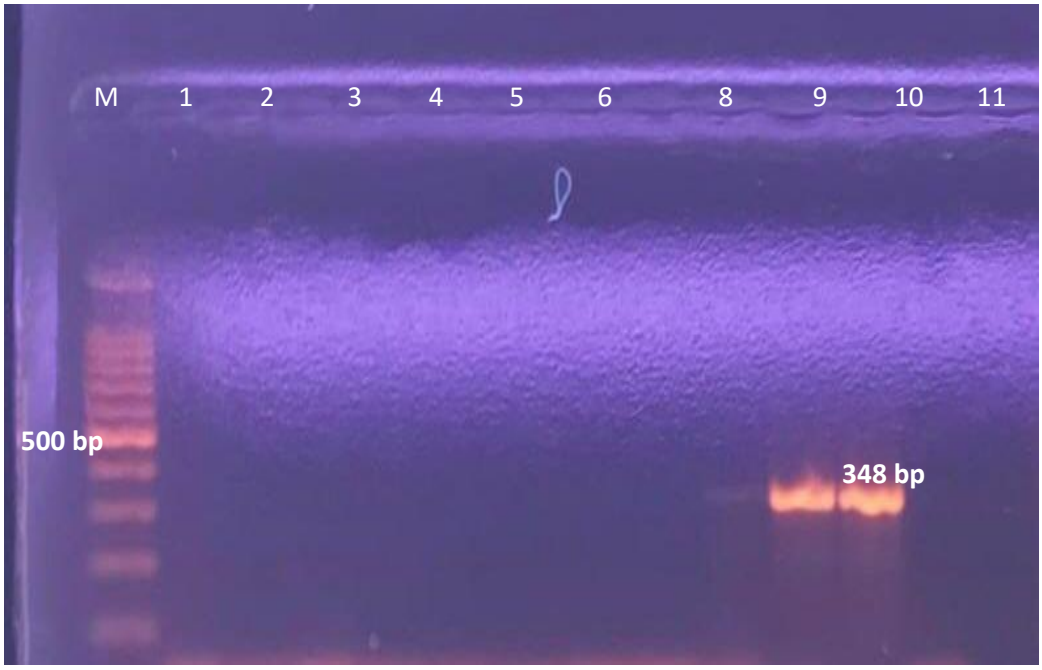


Figure (4-5):- Gel electrophoresis of aac(6')/aph(2'') gene for *S. aureus* bacteria in voltage (85V) time (70 minute)and 5µL of PCR product loaded in well (1-20). Lane M : DNA ladder (100 bp). All these sample are negative for the presence of this gene except well (9, 10) are positive.

Results of molecular experiments also demonstrated that *aph(3)-IIIa* gene had highest frequency in aminoglycoside – resistant isolates , the gene was *aac(6')/aph(2'')* less present , While we did not find the existence of *ant(4)-Ia* gene in the isolates. This indicates the presence of *aph(3)-IIIa* gene as the most important mechanism of resistance to aminoglycoside in *S. aureus* ,these results were not consistent with the results of studies of Liakopoulos *et.al*, in 2011 and Choi *et.al*, in 2003 those who reached their results that the gene *aac(6')/aph(2'')* was the highest and responsible for the mechanism resistance to aminoglycoside in *S. aureus* (Choi *et al.*, 2003 ; Shokravi *et al.*, 2015).

Looking at the studies regarding identification of genes responsible for resistance to aminoglycoside antibiotics, one can find that the highest prevalence belongs to *aac (6') Ie / aph (2")* gene and then *aph (3')* - *IIIa1* gene. These results have been reported by Shokravi *et al*2015. and Malek Hosseini *et al* .,2016 *aac (6') Ie / aph (2")* gene has the highest frequency in this study. However, *ant (4 ')* - *Ia1* with a prevalence of 24.77% is substituted with *aph (3')* - *IIIa1* with a frequency of 11.50%, which is not consistent with the results of the present study (Zhang *et al.*, 2010 ;Malek Hosseini *et al.*, 2016).These differences in results may be due to different geographic conditions, unique genotypic characteristics of bacteria, the location from which the sample is isolated and the type of samples. One of the most important factors that made the prevalence of antibiotic resistance, particularly aminoglycoside antibiotics, in this study different from other studies was the method, amount and period of using aminoglycoside antibiotics.

Genetic transfers can be prevent by modifying antibiotic administration pattern and not using various antibiotics in the process of treatment. Out of these 50 *Saphylococcus aureus* isolates that contain resistance genes, only 15 of them contained more than one resistance gene. Their distribution was 10 samples containing *aph(3)-IIIa* with *mecA* genes and only 5 containing *aph(3)-IIIa* with *aac(6')/aph(2")* genes.as in table (4-8).

Table (4-8): Genotyping distribution of aminoglycoside resistance genes with *mecA* gene for *S. aureus* in the study

Genes	**No. (100%)**
aph(3)-IIIa + mecA	10 (20%)
aph(3)-IIIa + aac(6')/aph(2")	5 (10%)

4.6. Effect of some medical plants on *S. aureus* resistance Isolated from Burn and Wound Infection

4.6.1. Effect of Curcumin

In well diffusion method various concentrations (25 ,50,100 and 200 mg\ml) from aqueous curcumin extract were test against *S. aureus* isolates and it showed inhibitory effects for only 20 from 63 *S. aureus* isolates in different concentrations by using serial dilutions . It was found that at concentration 200(mg/ml), the aqueous extract gave the highest inhibition effectiveness (Figure 4-6)

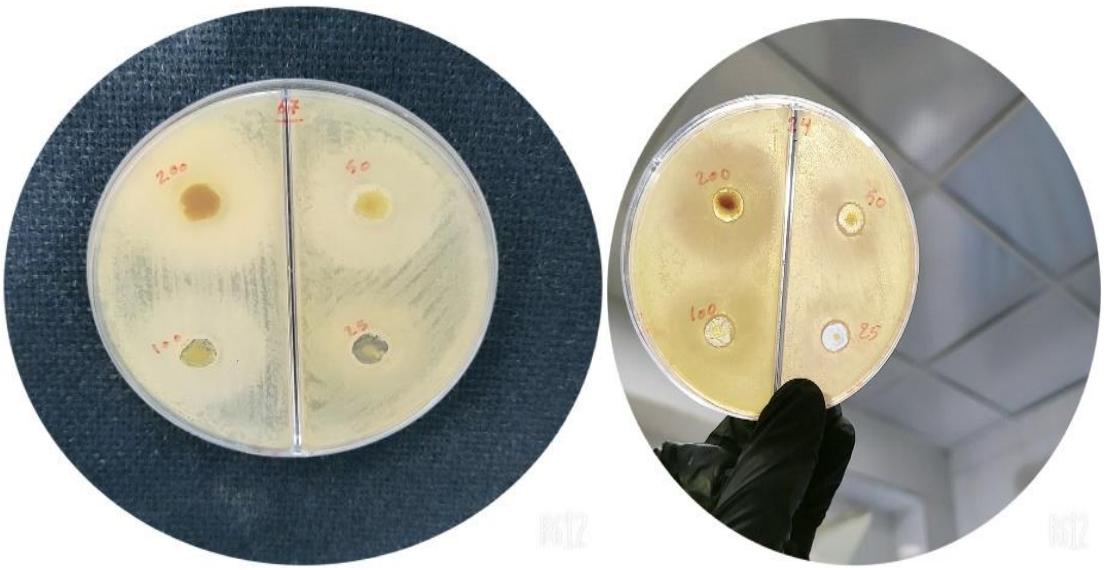


Figure (4-6) Inhibitory efficacy of aqueous curcumin extract on *S. aureus*

Curcumin may bind into proteins, thereby inhibiting the assembly of proteins. This, in turn, suppresses the formation of inhibition zone lead to inhibit cytokinesis and bacterial proliferation. The binding between curcumin and peptidoglycan on *S. aureus* cell wall could trigger damage on the cell wall membrane, leading to cell lysis of *S. aureus* (Teow *et al.*, 2016).

The results shown in Table (4-9) and Figure (4-7) show that the concentration of aqueous curcumin (200 mg/ml) has the highest effect on *S. aureus* bacteria, and all concentrations were significant at *P-value* 0.05. This suggests that the observed differences in bacterial growth inhibition between different curcumin concentrations were statistically significant. It suggests that the antimicrobial activity against *S. aureus* at high concentration of aqueous extract.

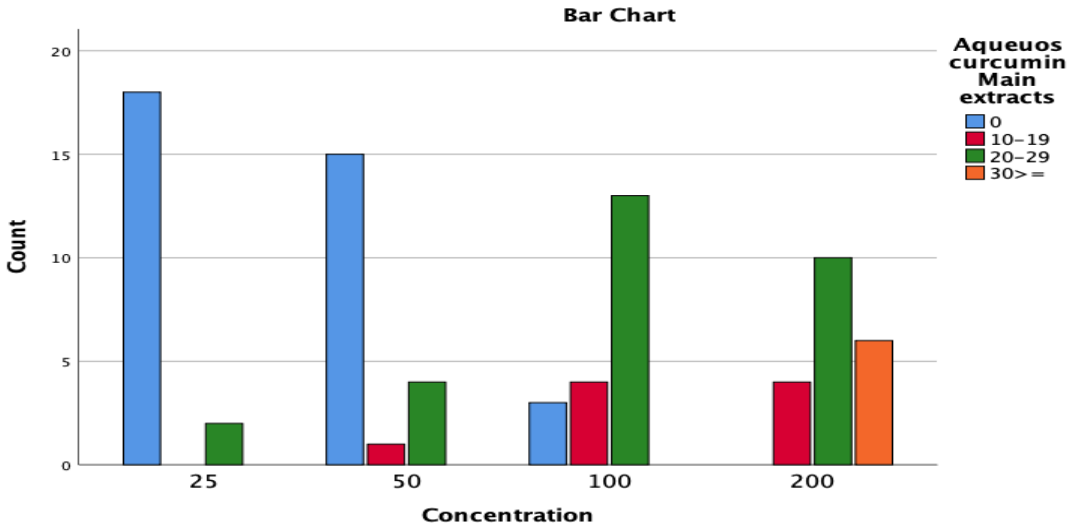


Figure (4-7): Distribution of Concentration with Aqueous curcumin.

Table (4-9) concentrations of aqueous curcumin extract

Concentration		Aqueuos curcumin Main extracts				
		0	10-19	20-29	30 ≥	Total
25	Count	18	0	2	0	20
	% within Concentration	90.0%	0.0%	10.0%	0.0%	100.0%
	% within Aqueuos curcumin	50.0%	0.0%	6.9%	0.0%	25.0%
	% of Total	22.5%	0.0%	2.5%	0.0%	25.0%
50	Count	15	1	4	0	20
	% within Concentration	75.0%	5.0%	20.0%	0.0%	100.0%
	% within Aqueuos curcumin	41.7%	11.1%	13.8%	0.0%	25.0%
	% of Total	18.8%	1.3%	5.0%	0.0%	25.0%
100	Count	3	4	13	0	20
	% within Concentration	15.0%	20.0%	65.0%	0.0%	100.0%
	% within Aqueuos curcumin	8.3%	44.4%	44.8%	0.0%	25.0%
	% of Total	3.8%	5.0%	16.3%	0.0%	25.0%
200	Count	0	4	10	6	20
	% within Concentration	0.0%	20.0%	50.0%	30.0%	100.0%
	% within Aqueuos curcumin	0.0%	44.4%	34.5%	100.0%	25.0%
	% of Total	0.0%	5.0%	12.5%	7.5%	25.0%
Total	Count	36	9	29	6	80
	% within Concentration	45.0%	11.3%	36.3%	7.5%	100.0%
	% within Aqueuos curcumin	100.0%	100.0%	100.0%	100.0%	100.0%
	% of Total	45.0%	11.3%	36.3%	7.5%	100.0%

The present study investigated the potential of using curcumin as an adjunct to antibiotic therapy for synergistic association against selected *S. aureus* drug sensitive and multidrug-resistant pathogens. When curcumin as an antibacterial agent enhances the action of other drugs, it may play a supportive role to improve the cellular uptake of other bacteriostatic agents by inhibiting the efflux pump rather than being the main antibacterial agent. The results came close to the studys of Oklo, 2023 and Anbari, *et al.,* 2022 who found the antibacterial activity shown by *C. longa* may be due to the presence of phytochemicals such as Alkaloids, Steroids, Saponins, Flavonoids, Phenols, and Tannins which were observed when the extract was analyzed phytochemically. *C.longa* showed potent activity against bacteria in this study and would make a more active and viable antibiotic (Oklo *et al.,* 2023). According to Moreno *et al.* the antimicrobial action of various phenolic complexes was associated to inactivation of different cellular enzymes which relied on the penetration rate of the substances into the cell and changes in the permeability of membrane. A change in the cell membrane permeability is the main factor in antimicrobial action of a particular compound. Phenolic compounds may completely disrupt the cellular membranes, affect the cellular integrity and cause ultimate cell death (Moreno *et al.,* 2006).

4.6.2. Effect of Berberine

In well diffusion method alcoholic berberine extract showed inhibitory effects for only 15 *S. aureus* isolates in different concentrations by using serial dilutions (500 , 250 , 125 , 62.5 mg/ml). It was found that at concentration 250 mg/ml, the alcoholic extract gave the highest inhibition effectiveness (Figure 4-8).

Figure (4-8) Inhibitory efficacy of alcoholic berberine extract on *S.aureus*

This figure shows that the concentration 250 mg/ml of alchoholic Barberein extract has the highest effect on *S. aureus* bacteria compared with the lower the concentration 62.5 g/ml and all concentrations were significant at *P-value* 0.05. as show in table (4-10).

Various clinical applications of berberine have been discovered, especially in antibacterial usage, which revealed that berberine with a quaternary nitrogen, polycyclic, and planar system could helpfully increase membrane permeability and strengthen the bind affinities with amino acids in biomolecules (Zhang *et al.*, 2018 ; Gaba *et al.*, 2021).

Table (4-10) Concentration with alcoholic Berberine Main extract

Concentration		Al-Choholic Berberine inhibition zone diameter				
		0	10-19	20-29	30 ≥	Total
63	Count	14	1	0	0	15
	% within Concentration	93.3%	6.7%	0.0%	0.0%	100.0%
	% within Choholic Barberein	100.0%	50.0%	0.0%	0.0%	25.0%
	% of Total	23.3%	1.7%	0.0%	0.0%	25.0%
125	Count	0	0	15	0	15
	% within Concentration	0.0%	0.0%	100.0%	0.0%	100.0%
	% within Choholic Barberein	0.0%	0.0%	48.4%	0.0%	25.0%
	% of Total	0.0%	0.0%	25.0%	0.0%	25.0%
250	Count	0	0	2	13	15
	% within Concentration	0.0%	0.0%	13.3%	86.7%	100.0%
	% within Choholic Barberein	0.0%	0.0%	6.5%	100.0 %	25.0%
	% of Total	0.0%	0.0%	3.3%	21.7%	25.0%
500	Count	0	1	14	0	15
	% within Concentration	0.0%	6.7%	93.3%	0.0%	100.0%
	% within Choholic Barberein	0.0%	50.0%	45.2%	0.0%	25.0%
	% of Total	0.0%	1.7%	23.3%	0.0%	25.0%
Total	Count	14	2	31	13	60
	% within Concentration	23.3%	3.3%	51.7%	21.7%	100.0%
	% within Choholic Barberein	100.0%	100.0%	100.0%	100.0 %	100.0%
	% of Total	23.3%	3.3%	51.7%	21.7%	100.0%

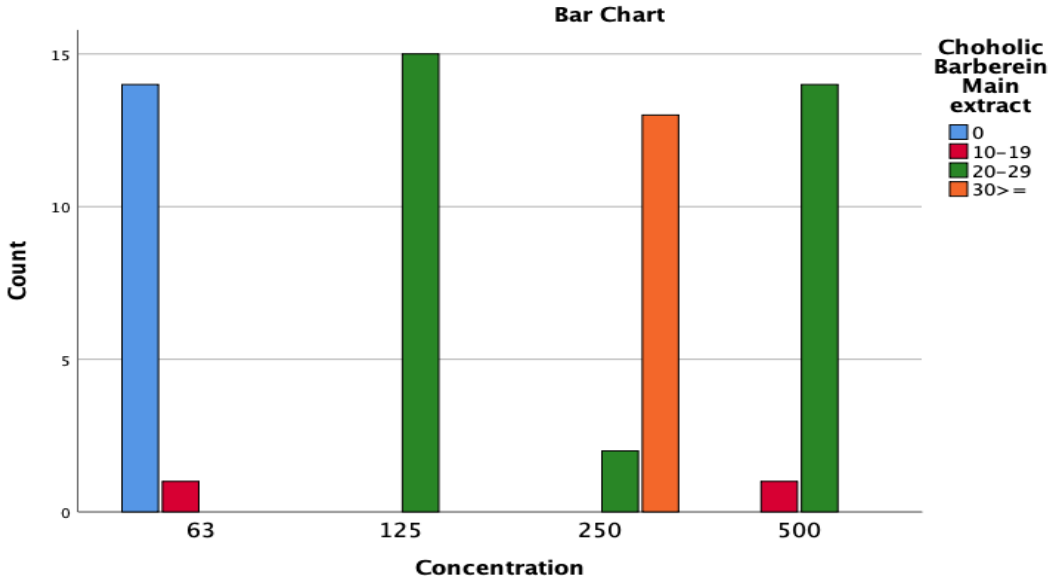


Figure (4-9):- Distribution of Concentration with alcoholic Berberine.

One study published in the Journal of Microbiology and Biotechnology in 2017 evaluated the antibacterial effects of berberine against clinical isolates of *S. aureus* (Yang, *et al.*, 2017). The results demonstrated that berberine exhibited significant inhibitory activity against the tested strains. Another study published in the Journal of Natural Products in 2019 found that berberine was effective in inhibiting the growth of *S. aureus* biofilms, which are communities of bacteria that can be particularly resistant to antibiotics (Tan, *et al.*, 2019). The exact mechanism by which berberine exerts its antimicrobial effects on *S. aureus* is not fully understood, but several mechanisms have been proposed. It is believed that berberine can disrupt the bacterial cell membrane, interfere with DNA replication and protein synthesis, and inhibit enzymes necessary for bacterial survival (Sharma, *et al.*, 2023).

From Table (4-11), show the convergence of effect between both curcumin and berberine, with the highest average effect reaching 25.25 ± 4.089 for curcumin and 31.60 ± 2.501 for berberine.

Table (4-11) Concentration and Main comparison between aqueous curcumin and alcoholic Berberine.

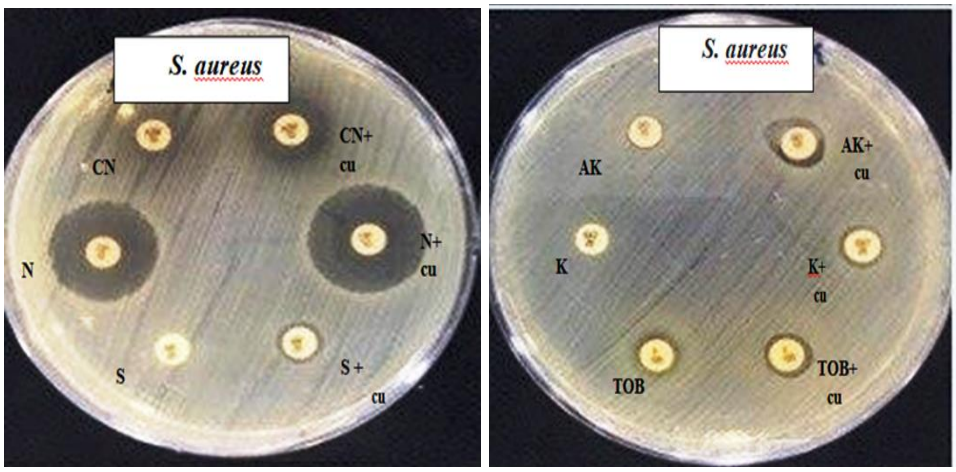
Aqueuos curcumin Concentration Main extracts			
Concentration mg/ml	**N0.**	**Mean ± SD**	***P-Value***
25	20	2.40 ± 7.387	.473*
50	20	4.85 ± 8.964	.394*
100	20	18.90 ± 9.055	.213*
200	20	25.25 ± 4.089	.136**
Al-Coholic Berberine Concentration Main extract			
Concentration mg/ml	**N0.**	**Mean ± SD**	**P-Value**
63	15	1.00 ± 2.803	.495*
125	15	22.93 ± 2.120	.181*
250	15	31.60 ± 2.501	.064**
500	15	22.60 ± 2.414	.180*

***P value* is strong significant

4.6.3. Synergistic effect of curcumin and berberine with Aminoglycoside antibiotic

The synergistic effect of medical plants (curcumin and berberine) was investigated with 6 Aminoglycoside antibiotics against the higher Aminoglycoside resistance isolates of *S. aureus* .as in figure (4-10).

A.



B.

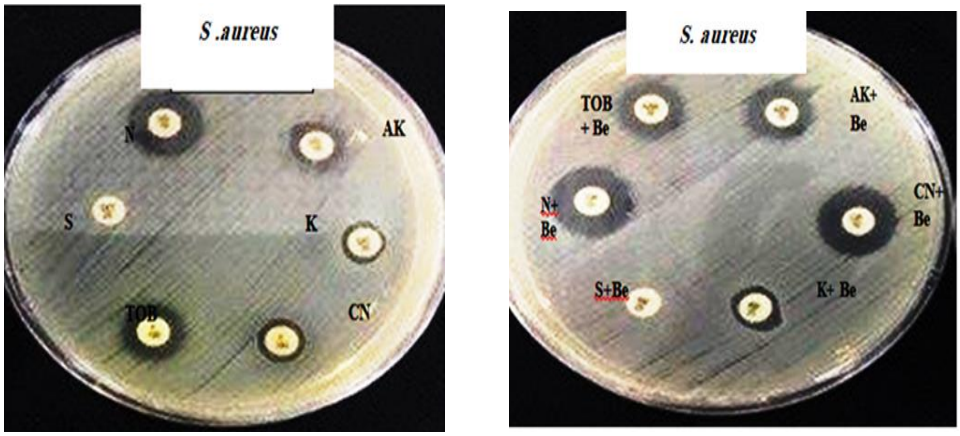


Figure (4-10): The synergistic effect of AK, K, TOB, S, CN and N with plant extract increase against Aminoglycoside resistance isolates of *S. aureus* A: - with Curcumin B: - with Berberine.

The same situation was observed for the synergistic effect of AK, K, TOB, CN and N with berberine increase against Aminoglycoside resistance isolates of *S. aureus* except S which did not has any synergistic action between its and berberine. Based on its broad-spectrum antibacterial activity, using curcumin in combination with other existing drugs to potentiate their antimicrobial activity seems to be a promising approach. The application of the combination of herbal drugs with antibiotics has

already been suggested, especially for antibiotic-resistant strains (Zhou *et al.,* 2017). Successful combination of the antibiotic with curcumin could reduce the dose of antibiotics and the compounds, by interacting with different targets in the bacterial cell can overcome its resistance mechanisms (Zheng *et al.*, 2020).

Antibacterial combinations are used widely, although most infections in patients with normal defenses can be treated with a single antibacterial agent. Few reasons justify the use of antibacterial combinations: (1) broad-spectrum coverage for the initial therapy of severely infected patients; (2) poly-microbial infections; (3) prevention of selection of resistant microorganisms when a high mutation rate of the causal organism exists to the antibiotic indicated; (4) reduction of dose related toxicity – this concern is rare and mostly of historical interest, related to the use of sulfonamides; and (5) antibacterial synergistic activity (Acar , 2000) .

Synergism is associated with the generation of hydroxyl radicals, alteration of protective cellular functions and an anti-biofilm potential. The combination of antibiotics with medical plants is more effective for enhancing antibiotic efficacy in comparison with the action of antibiotics when used in clinical practice. The combination involves reduced development of bacterial resistance, reduce the duration of treatment and reduce antibiotic dose requirements (Hwang *et al.,* 2012).Curcumin Natural producthas also been found to possess many beneficial biological activities, including antioxidant, antimicrobial, antitumor, and anti-inflammatory properties as well as a potent inhibitory effect on nuclear factor-kappa B (Sintara *et al*,2010).

In more than one study, the synergistic role of antibiotics as an antibacterial has been demonstrated, and one of these studies is a study of Sasidharan *et al*, 2014 who investigated the synergistic activity of curumine with three clinically used third generation cephalosporin antibiotics (cefaclor, cefodizime, and cefotaxime) against bacteria associated with diarrhea.

In the present study curcumin synergistic recorded antibacterial activity at higher concentration. This is in accordance with the previous report by (Gunes *et al*, 2016). However, for many antibiotics, such as aminoglycosides, it is not feasible to significantly increase the dose of the antibiotic because of toxic side effects. In these circumstances, benefit for the patient could be achieved by enhancing the effect of the antibiotic against resistant bacteria (Sasidharan *et al.*, 2014). Here, through present study clearly showed that curcumin enhances the activity of Amikacin, Gentamicin, Streptomycin, Neomycin, Tobramycin, and Kanamycin. Most interestingly, the bactericidal activities of the tested drugs were significantly enhanced. Synergistic effect of curcumin with antibiotics is previously reported against methicillin-resistant *S. aureus* and *C. albicans* (Muna *et al*, 2013).

Berberine is an isoquinoline alkaloid widely used in the treatment of microbial infections. Recent studies have shown that berberine can enhance the inhibitory efficacy of antibiotics against clinical Aminoglycoside resistant *S. aureus*. In one of the studies that conducted by Chu *et al*,2016 where he demonstrated the synergistic role of berberine with a wide spectrum of antibiotics against clinical multi-drug resistant isolates of methicillin-resistant *S.aureus* (MRSA) explaining that berberine isoquinoline alkaloid so it has Role in inhibited methicillin-resistant *S.aureus* (Gao ,2014).

In a study previously presented by Ahmadi *et al.,* in 2022 who concluded the effect of berberine alone and in combination with the ciprofloxacin and thioridazine berberine in combination with ciprofloxacin reduced the MIC of ciprofloxacin between zero and 2 fold. According to Ahmadi *et al.*, in 2022 studies, gram-positive bacteria were more sensitive to berberine than gram-negative bacteria. however, in study of, berberine alone had no inhibitory effect on *A. baumannii*, which may be due to the use of bacterial strains from different sources or due to the use of different berberine derivatives, which may affect the MIC (Gao *et al.,* 2018) . Efflux transporter mediated bacterial resistance to different and curcumin and berbrine may inhibit this efflux pump system antibiotics (Ryan *et al.*,2020). Medical plant have drawn the attention of many scientists because of its extensive pharmaceutical properties (Hsu and Cheng ,2017).

4.7. Efficiency of curcumin and berberine extract on cell lines

4.7.1. Effect of Curcumin on Breast cancer cell lines (MCF-7) and normal human liver cell (WRL68) cell lines

The table (4-12) shows the cell viability percentages of MCF-7 and WRL68 cell lines treated with different concentrations of curcumin (400, 200, 100, 50, 25, 12.5 µg/ml) extract for 48 h. incubation period. Curcumin were used to evaluate their effects of on breast cancer MCF-7 cells and normal hepatic cell WRL68 cells compared to Dimethyl sulfoxide (DMSO) that used as control as in figure (4-11) and figure (4-12), cytotoxicity in the presence of independent compound at different concentrations was evaluated by (MTT) method, For MCF-7 cell line, the cell viability percentages range from 95.18% ± 1.280% at the lowest concentration of 12.5 µg/ml to 37.62% ± 3.451% at the highest concentration of 400 µg /ml, with IC50 value for the curcumin extract is

reported as 269. This indicates that the concentration of the curcumin required to inhibit 50% of cell viability in MCF-7 cell lines is 269 µg/ml. While the cell viability percentages of WRL68 cell lines after treatment with different concentrations of curcumin range from 95.95% ± 1.028% at the lowest concentration of 12.5 µg/ml to 77.74% ± 3.210% at the highest concentration of 400 µg/ml with IC50 value for the curcumin is reported as 919. The cell survival rate depended on curcumin concentration and incubation duration.

Table (4-12):- The viability of MCF-7 and WRL68cell line under Curcumin extract exposure represented with mean and standard deviation.

curcumin	cell viability %						IC50
	Concentration of curcumin extract (M ± SD)						
	400 µg /ml	200 µg /ml	100 µg /ml	50 µg /ml	25 µg /ml	12.5 µg /ml	
MCF-7	37.62±3.451	45.53±4.245	69.33±3.151	90.70±3.183	95.72±3.183	95.18±1.280	**269**
WRL-68	77.74±3.210	82.75±2.842	92.44±2.713	95.33±1.183	95.22±0.8209	95.95±1.028	**919**
DMSO	92.41±1.882	94.42±0.886	92.44±2.713	95.33±1.182	95.22±0.821	95.95±1.028	

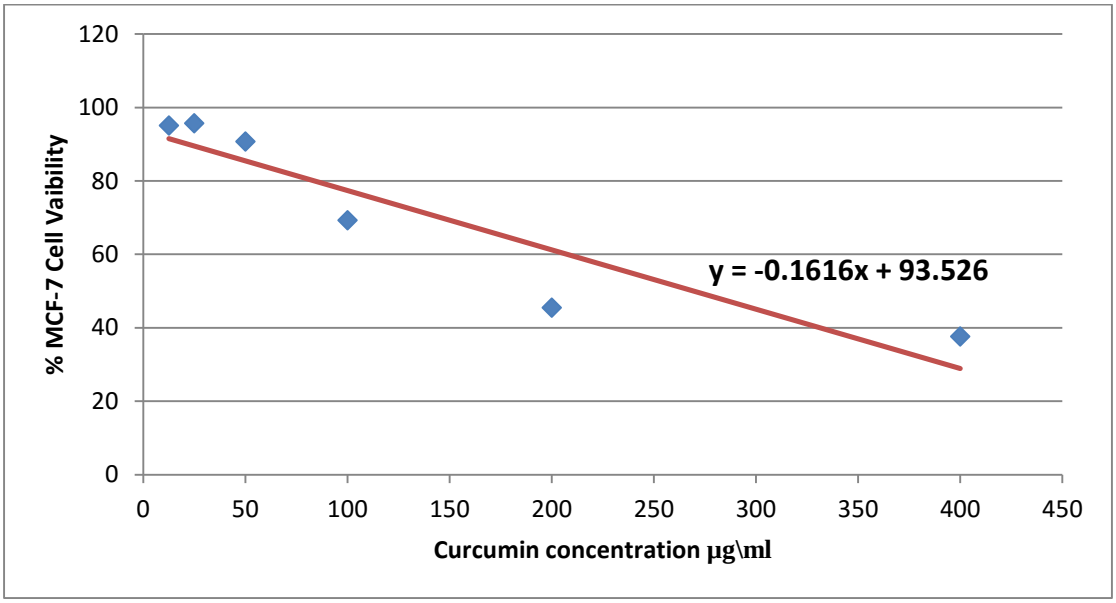


Figure (4-11): Percent of human Breast cancer cell line (MCF-7) viability under curcumin exposure compared with DMSO as control.

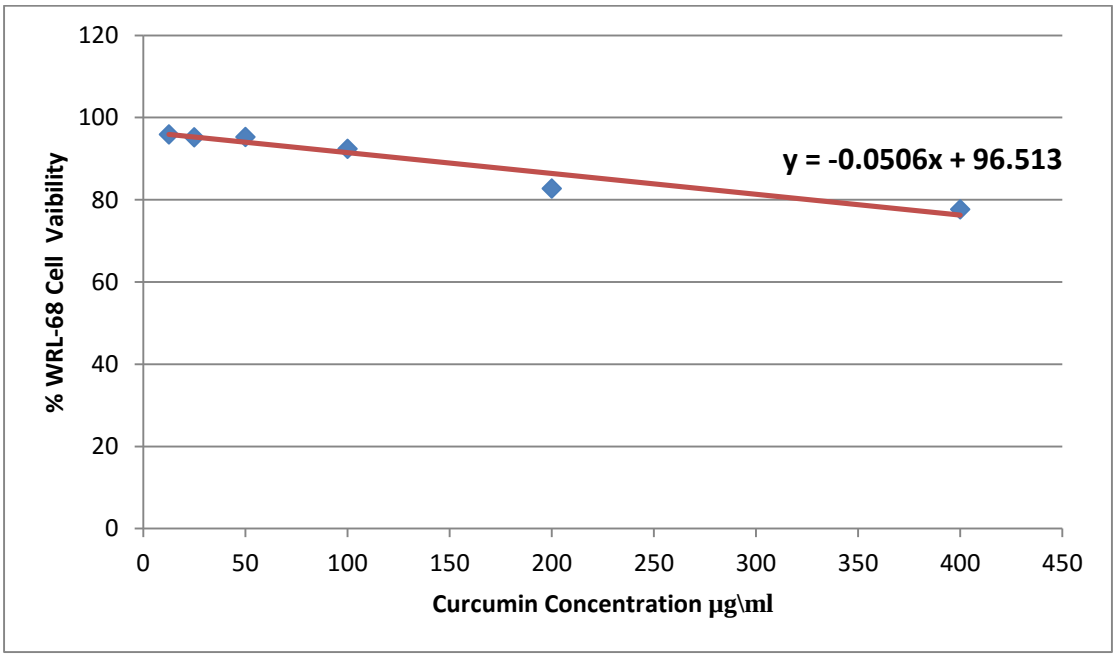


Figure (4-12):- Percent of human hepatic cell line (WRL68) viability under curcumin Extract exposure compared with DMSO as control.

Curcumin is a polyphenolic compound in turmeric (*Curcuma longa*) rhizomes that displays potential effects against a wide variety of chronic diseases including cardiovascular, inflammatory, metabolic, neurological and skin conditions; various infectious diseases; and cancer (Kunnumakkara *et al.*, 2017 ; Bashang and Tamma 2020). Curcumin inhibits breast cancer cell proliferation by inducing cell cycle arrest and p53-dependent apoptosis, altering the expression of signaling proteins, down regulating transcription factors, and inhibiting tumor growth and angiogenesis (Song *et al.*, 2019). In study conducted by Kunwar *et al.*, 2008, the effect of curcumin toxicity on two cancer cell lines (MCF-7 and EL-4) was investigated in 2D culture for 20 h by the spectroscopic fluorescence method. Their results showed that as curcumin concentration increased, the cell mortality rate also increased, these results are compatible with results of this study .

Also, in 2017, Mansorabadi *et al.*, investigated the toxicity effects of curcumin on mouse breast cancer cell line by the MTT method. In the above mentioned study, concentrations of 5-40 μM of curcumin were added to the cells, and after incubation for 24 and 48 h, the viability of the cells was evaluated by the MTT method. The IC50 of the curcumin in 24 and 48 h was 21 ± 0.3 μM and 8.14 ± 0.4 μM, respectively. While the effect of curcumin extract on normal hepatic cells (WRL68) was very small compared to its effect on cancer cells. Curcumin has a strong impact on immune system functions by increasing the number of $CD8^+$ Tcells, stimulating the Treg switch to Th1 cells, reducing the number of Treg cells, inhibiting Foxp3 and inducing IFN-γ expression. Like phenolic acids, curcumin also inhibits melanogenesis (Tu *et al.*, 2012), by inhibiting tyrosinase activity and expression of melanogenesis-related molecules such as TRP-1 and TRP-2. Therefore, curcumin may share at least part of

the mechanism of action with some phenolic acids .A direct action of curcumin on gut microbiota has also been reported (Zhu *et al.,* 2022), indicating an additional way it may act to control immune response.

4.7.2. Effect of Berberine on Skin cancer cell lines (A431) and normal human keratinocyte cell lines (HaCaT).

In this study , table (4-13) shows the cell viability percentages of A431 and HaCaT cell lines treated with different concentrations of berberine extract (400 ,200,100,50,25,12.5 µg/ml) for 48 h. incubation period. Berberine were used to evaluate their effects of on skin cancer A431 cells and normal keratinocyte HaCaT cells compared to Dimethyl sulfoxide (DMSO) that used as control as in figure (4-13) and figure (4-14), cytotoxicity in the presence of independent compound at different concentrations was evaluated by (MTT) method.

For berberine on A431 cell lines , the cell viability percentages range from 95.41% ± 1.414% at the lowest concentration of 349.2 µg/ml to 47.96% ± 3.966% at the highest concentration of 400 µg/ml. compared to DMSO, the cell viability percentages range from 95.41% ± 1.414% to 91.96% ± 4.239% across different concentrations. And all are significant at *P-value* 0.05.

The cell viability percentages of HaCaT cell lines after treatment with different concentrations of berberine range from 94.91% ± 2.199% at the lowest concentration of 12.5 µg/ml to 74.58% ± 6.078% at the highest concentration of 400 µg/ml. For DMSO, the cell viability percentages range from 94.91% ± 2.199% to 89.91% ± 7.578% across different concentrations. The IC50 value for the berberine is reported as 807. This

indicates that the concentration of the berberine extract required to inhibit 50% of cell viability in HaCaT cells is 807 .

Table (4-13):- The viability of A431 and HaCaT cell line under berberine extract exposure represented with mean and standard deviation.

Berberine	cell viability %						IC50
	Concentration of barbrine extract (M ± SD)						
	400	200	100	50	25	12.5	
A431	47.96± 3.966	67.17± 1.076	77.62± 2.412	90.59± 1.802	94.79± 1.335	95.41± 1.414	**363**
HaCaT	74.58± 6.078	86.03±0.853	92.13±1.557	96.18±1.252	96.95±1.142	94.91±2.199	**807**
DMSO	89.91±7.578	95.37±1.202	93.13±1.267	96.18±1.252	96.95±1.142	94.91±2.199	

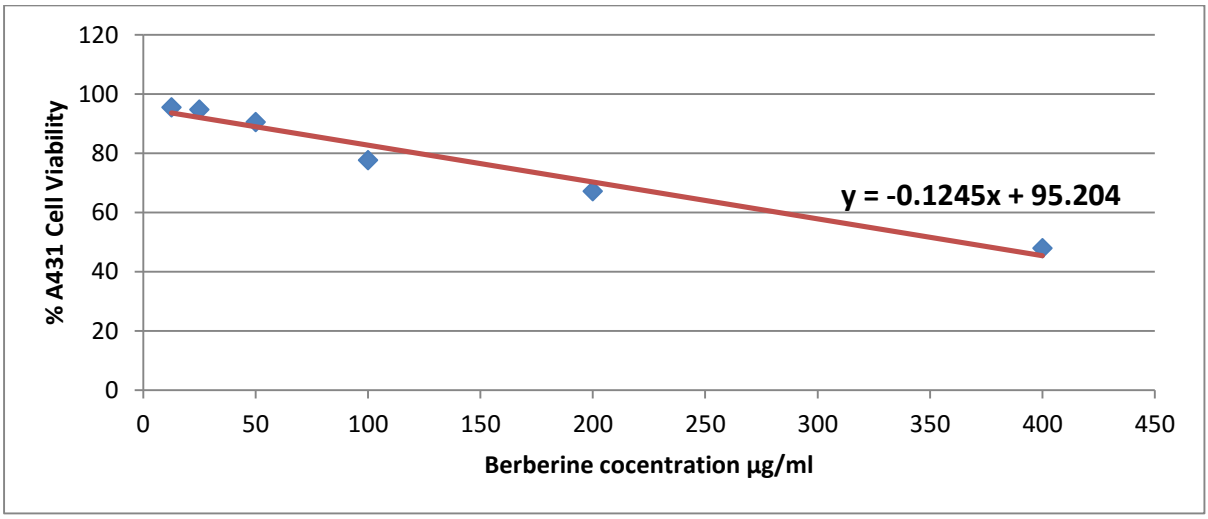


Figure (4-13): Percent of human skin cancer cell line (A431) viability under berberine extract exposure compared with DMSO as control

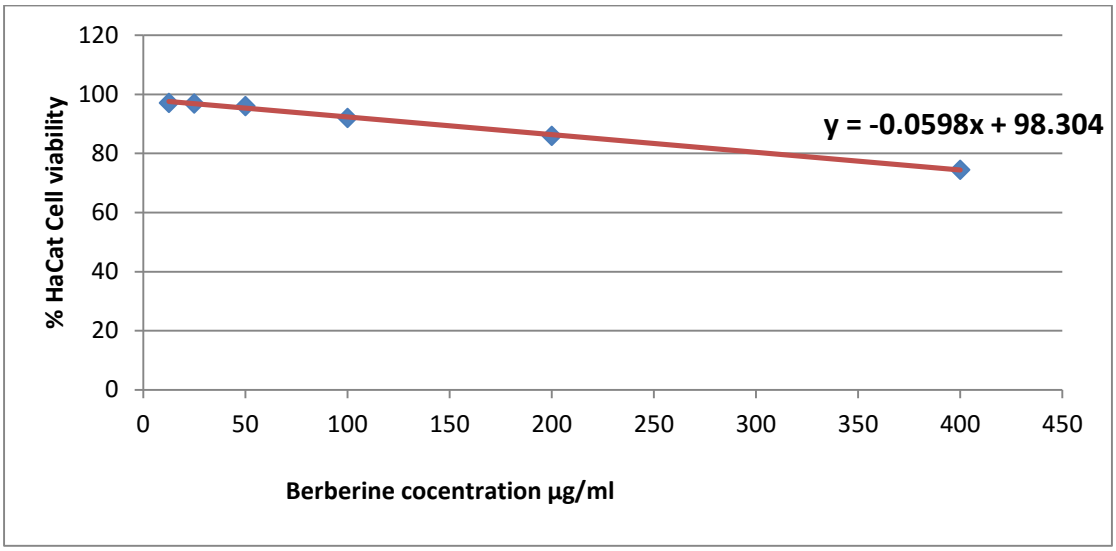


Figure (4-14): Percent of human keratinocyte cell line (HsCat) viability under berberine extract exposure compared with DMSO as control

Many potential cancer-protective agents can be broadly categorized as blocking agents, which impede the initiation stage, or suppressing agents, which arrest the promotion and progression of tumor, presumably by affecting or disturbing crucial factors that control cell proliferation, differentiation, or apoptosis (Hsu *et al.*, 2007). Berberine shows proapoptotic effects in many cancer cell lines and non-tumor cells, including A431 (Mantena *et al.*, 2006).

BER was found earlier to suppress the proliferation of skin squamous carcinoma cells (A431) in a time- and concentration-dependent manner. Moreover, BBR treatment induced different biochemical changes, such as loss of the membrane potential of mitochondria, cytochrome-c release into the cytosol, and cleavage of the poly (ADP) ribose polymerase. Results revealed that BER induces apoptotic conditions and inhibits skin squamous carcinoma cells (Li *et al.*, 2015).

Berberine inhibits DNA topoisomerase I and II in biochemical system (Kim *et al.,* 1998). The different concentrations used in different studies may account for the conflicting information in the literature, as berberine at low doses (12.5–50 µmol/l) is concentrated in mitochondria and promotes G1 arrest, whereas higher doses (over 50 µmol/l) result in cytoplasmic and nuclear accumulation and G2 arrest (Serafim *et al.,* 2008). In fact, as early as 1996, berberine was found mainly in cytoplasm during berberine-induced (100 mg/ml) cell cycle G2/M arrest, whereas it was highly concentrated in nuclei in the induction of apoptosis under high dose (200 µg/ml) (Yang *et al.,* 1996). In addition, different cell lines exhibit significantly different sensitivities to this alkaloid, as discussed above. Recent detailed knowledge on molecular carcinogenesis provided the potential for therapeutic intervention in cancer by specifically targeting and sensitizing cancer cells to apoptosis (Bremer *et al.,* 2006).

Thus, results of current study are consistent with the results of the study in 2006 presented by Mantena *et al., who* assess the effect of berberine on human skin cancer cells, by determined the effect of berberine on the proliferation and cell viability of the A431 cells using MTT assay and the cytotoxic effect or cell death by using trypan blue exclusion assay. They found that the cells were treated with varying concentrations of berberine (0, 5, 10, 15, 25, 50 and 75 µM) for 12, 24, 48 and 72 h. The treatment of A431 cells with berberine resulted in a significant reduction in cell proliferation/viability as assessed by MTT assay. The dose-dependent reduction in viability of the cells ranged from 12–58% ($P < 0.05–0.01$) after 48 h.

4.8. Gene expression

4.8.1. Gene expression of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene after Exposure to plant extracts

The present study used quantitative RT-PCR to assess the mRNA expression of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene and compare it after *S. aureus* bacteria were incubated with curcumin and berberine at a concentration of (3 mg /ml) and (125µg/ml) respectively for 24 hours. The fold change in gene expression was calculated using relative quantification. This is dependent on the normalization of Ct values when calculating the Ct, which is the difference between the mean Ct values of each replicate of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* cDNA amplification and the housekeeping 16S rRNA .

Comparative Ct method was also referred to as the fold change= $2^{-\Delta\Delta}$ Ct approach of (Livak and Schmittgen 2008), which was a golden equation technique for comparing gene expression in various samples based on relative gene expression data. Each sample was compared to an internal control gene in both treated and untreated samples to establish that the observed differences were due to changes in target gene expression rather than mRNA quality or quantity.

In the control, the fold of gene expression for *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* cDNA amplification was (1.00),the expression values of these genes with curcumin extract were (0.55 , 0.69 , 0.24 respectively) had the lowest values compared to the control, indicating that they function as an inhibitor. The results are Figure (4-16).

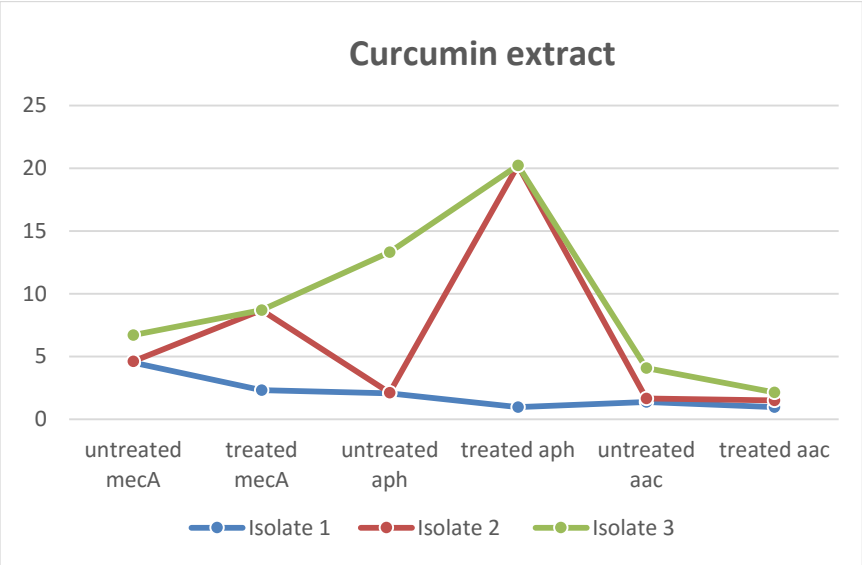


Figure (4-16): The Amplification Plots of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene after and before the Curcumin extract .

While in berberine extract the control, the fold of gene expression for *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* cDNA amplification was also (1.00),the expression values of these genes with berberine extract were (0.92 , 0.91 , 0.69 respectively) had the lowest values compared to the control, indicating that these genes were reduced by (0.92 , 0.91 , 0.69 respectively) times in the treated group compared to the untreated group, indicating that they function as an inhibitor. Figure (4-17).

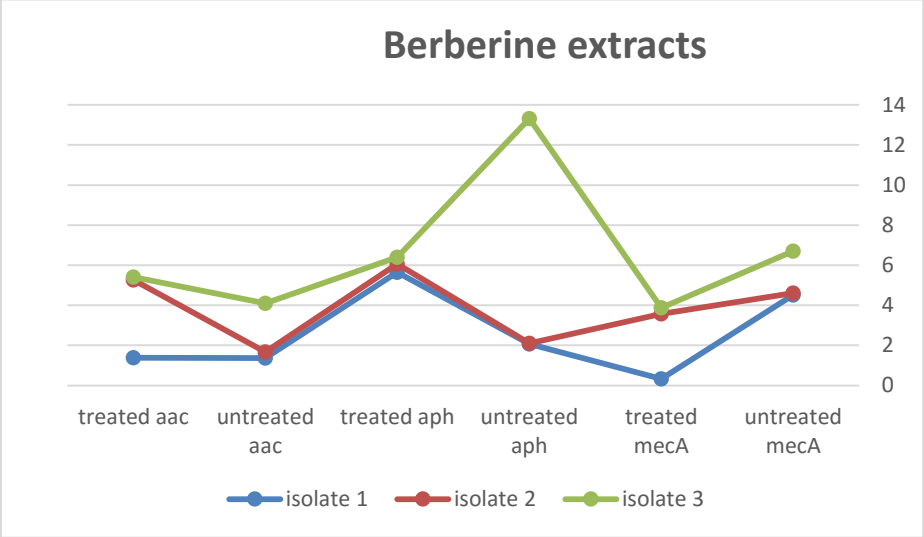


Figure (4-17): The Amplification Plots of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene after and before the Berberine extract

Due to the greater abundance of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene, the expression of these genes were measured under the influence of curcumin and berberine , and by using the Real-time PCR technique, the reproduction curve is shown in (appendix 4) .

The relative expression of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* gene in isolates treated with Curcumin and berberine showed a significant change compared to the isolates untreated and based on the results, which indicates the significance of the difference in the expression of these genes between the treated and untreated groups.

Table (4-14):- Gene expression for *S. aureus* isolates treated with curcumin and berberine extract and with *aph(3)IIIa* gene

	houskeeping gene 16SrRNA			interested gene *aph(3)-IIIa*						
sample	Ct 1	Ct 2	average Ct	Ct 1	Ct 2	average Ct	dCT	ddCT	fold change	
40			28.26			19.13	-9.13	-1.046667	2.066	
50			28.31			24.76	-3.55	4.5333333	0.043	
64			28.41			16.84	-11.57	-3.486667	11.210	1.00
							-8.0833333			
40 C			36.36			28.31	-8.05	0.0333333	0.977	
50 C			32.11			19.76	-12.35	-4.266667	19.248	0.55
64 C			38.01			36.76	-1.25	6.8333333	0.009	
40B			30.21			19.63	-10.58	-2.496667	5.644	
50B			22.01			15.17	-6.84	1.2433333	0.422	0.92
64B			28.03			21.56	-6.47	1.6133333	0.327	

Table (4-15):- Gene expression for *S. aureus* isolates treated with curcumin and berberine extract and with *aac(6')/aph(2'')* gene

	houskeeping gene *16SrRNA*			interested gene *aac(6')/aph(2")*						
sample	Ct 1	Ct 2	average Ct	Ct 1	Ct 2	average Ct	dCT	ddCT	fold change	
40			28.96			37.34	8.38	-0.456667	1.372	
50			28.31			38.88	10.57	1.7333333	0.301	
64			28.23			35.79	7.56	-1.276667	2.423	1.00
							8.83666667			
40 C			27.96			36.83	8.87	0.0333333	0.977	
50 C			27.05			36.81	9.76	0.9233333	0.527	0.69
64 C			27.43			36.89	9.46	0.6233333	0.649	
40B			23.23			31.59	8.36	-0.476667	1.392	
50B			20.56			27.44	6.88	-1.956667	3.882	0.91
64B			24.03			35.73	11.7	2.8633333	0.137	

Table (4-16):- Gene expression for *S. aureus* isolates treated with curcumin and berberine extract and with *mecA* gene

		housekeeping gene *16SrRNA*			interested gene *mec A gene*						
sample	Ct 1	Ct 2	average Ct		Ct 1	Ct 2	average Ct	dCT	ddCT	fold change	
40			25.12				18.28	-6.84	-2.173333	4.511	
50			28.31				26.88	-1.43	3.2366667	0.106	
64			25.22				19.49	-5.73	-1.063333	2.090	1.00
								-4.6666667			
40 C			25.21				19.33	-5.88	-1.213333	2.319	
50 C			25.35				18.01	-7.34	-2.673333	6.379	0.24
64 C			25.61				31.03	5.42	10.086667	0.001	
40 B			25.15				22.06	-3.09	1.5766667	0.335	
50 B			25.65				19.29	-6.36	-1.693333	3.234	0.69
64 B			25.11				22.17	-2.94	1.7266667	0.302	

C: - curcumin B: - berberine

In study of Mireshghi *et al.*, 2023 revealed that nanotechnology was used to improve the effectiveness of curcumin, which improved the performance of curcumin on *Shigella dysenteriae* and reduced the minimum growth inhibitory concentration compared to Kareem's research to 225 µg/mL (Kareem *et al.*, 2020). Therefore, the nanoization of curcumin particles is more effective than curcumin. In this regard, the effectiveness of nanoparticle curcumin was supported by a study presented by researchers Shariati *et al.*, in 2019 who showed Nano-curcumin represents a significant advance as an antimicrobial agent against *MDR P. aeruginosa* strains and burn infections.

Their synthesized nanoparticles destroyed biofilm, making them good candidates for future studies in terms of anti-biofilm agents, disinfecting surfaces, and deregulation of virulence- gene expression. Curcumin nanoparticles have good stability and solubility; therefore, the difficulties inherent in curcumin administration can be circumvented.

In 2018 another study presented by researchers Jaberi *et al.,* who examine the effect of curcumin on expression of norA gene in ciprofloxacin resistant *S. aureus.* The expression of NorA was significantly decreased in this isolated ($P< 0.05$) when it was treated with curcumin extract compared with absent of curcumin. The Pfafi method was used for analyse of results, and found that in more than 82% of sampls, curcumine redused the rate of expression of NorA gene. As well as in more than half of strains NorA expression reduction was more than 10 times in the present of curcumin compare the lack of curcumin. In 18% of samples, decrease was more than 100 times, and this is a great result. Only in 2 of the samples, we have the increase in exprestion of NorA, while in one them increase is very minor and it is negligible.Through the results of the current study, it was found that the berberine extract has an effective inhibition of isolates that contain *aph(3)-IIIa , aac(6')/aph(2'')* and *mecA* genes in compared isolates without treatment with the extract.

Studies such Kaatz *et al.,* 2004 ; Yu *et al.,* 2005 and Wang *et al.,* 2008 have shown that berberine not only possessed bactericidal activity but also an antibiotic enhancing property in *S. aureus*. Surprisingly, even though berberine is the substrate of the NorA efflux pump of *S. aureus*, its presence alone has not altered the expression of the norA gene. BER had been used in clinic for decades; and its various antibacterial mechanisms

had been fascinating. And this feature made it difficult to develop drug resistance (Jin *et al.,* 2010) .

In 2019, a study by Tan *et al.*, was conducted to investigate the antimicrobial activity of berberine against clinical derived PJI-related *S. aureus* propose the reason could be that berberine does not exert antimicrobial efficacy against staphylococci by direct killing, but rather, by inhibiting the bacteria. Thus, certain amount of bacteria is able to survive and persist in high berberine concentration environment, and as berberine gradually depletes the survivors regain a favorable environment. This infers that although berberine displays excellent inhibiting effects against *S. aureus* while used alone berberine is not sufficient to control S. aureus infections. Therefore, they suggest that berberine be used as an ancillary drug in combination with other antibiotics in *S. aureus* related PJI control, since berberine shows remarkable synergy effects with a wide range of antibiotics **(**Yu *et al.*, 2005 and Zhou *et al.*, 2015).

5.1. Conclusions

- *Staphylococcus aureus* was the most dominant bacterial species among the isolates taken from burn and wound patients. *S.aureus* recorded a pattern of resistance to aminoglycoside antibiotics, and the highest resistance was to kanamycin followed by tobramycin while the least resistance appeared in gentamicin and amikacin .
- TCP method showed that *S. aureus* isolated from burn wound have high degree of biofilm forming ability.
- Isolates of *staphylococcal* bacteria that showed resistance to aminoglycosides antibiotics showed aminoglycosides genes, *aph(3)-IIIa* gene had highest frequency in aminoglycoside – resistant isolates than the other genes and therefore it is responsible for the resistance shown by the isolates towards antibiotics.
- Curcumin and berberine showed antibacterial activity against *Staphylococcus aureus*, aqueous curcumin at the concentration 200 mg/ ml gave the highest inhibition activity for bacteria, while the alcoholic berberine gave the inhibitory effectiveness at the concentration 250 mg/ml.
- The effectiveness of curcumin and berberine as anti-cancer through induces cytotoxic effect in human breast cancer cell lines (MCF-7) and human epidermoid carcinoma (A431) cells respectively, but not or few in normal human hepatic cell line (WRL68) and human epidermal keratinocytes (HaCaT) respectively.
- The gene expression of curcumin and berberine extracts led to down-regulation in the gene expression of the studied genes which include *mecA* , *aph(3')-IIIa1 aac(6')Ie/aph(2'')gene* , compared to housekeeping gene 16SrRNA.

5.2. Recommendation

- Isolation and identification of other bacterial species besides staphylococcus bacteria for burn and wound patients
- Determination of the burn degree of isolates taken from burn patients.
- Conducting a sensitivity test against antibiotics for more than one class of antibiotics, such as beta-lactams and macrolides besides aminoglycosides antibiotics.
- Testing the effectiveness of other phytochemicals on bacteria isolated from burns and wounds patients.
- Testing the effectiveness of curcumin and berberine as anti- biofilm and anti-hemolysis against pathogenic bacteria.
- Study the effect of curcumin and berberine on other cell lines.
- Further studies may be understand the mechanisms of beberine's effect on the cell cycle in more detail and to determine the genetic and protein structure responsible for the sensitivity of cells to this compound.
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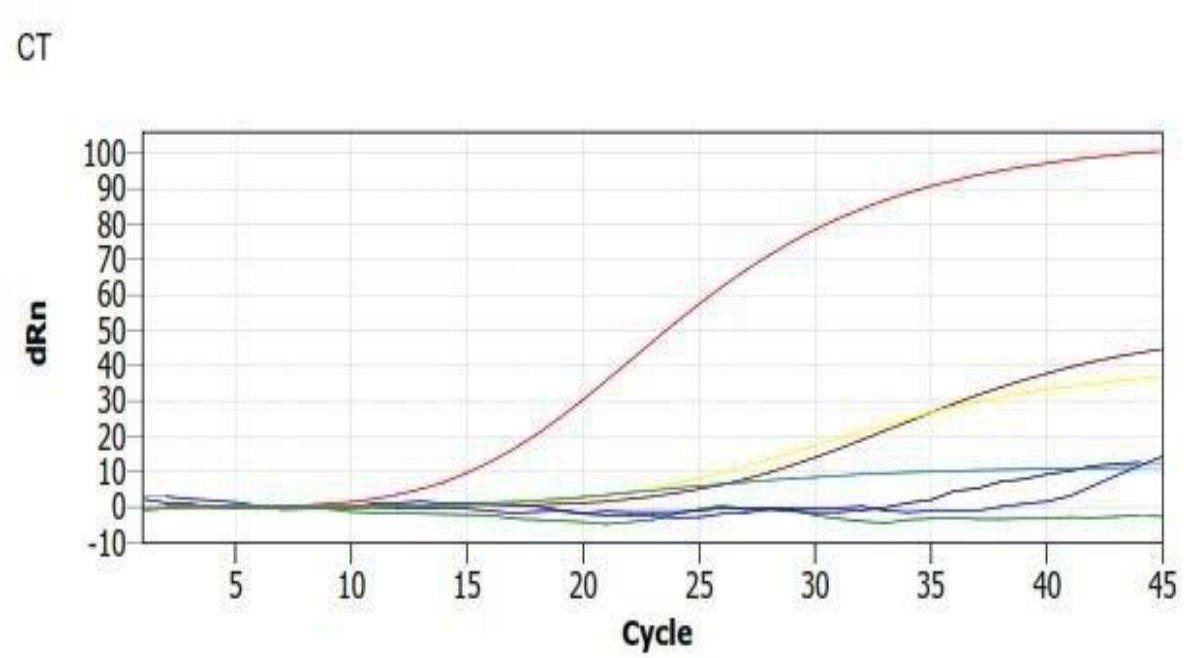
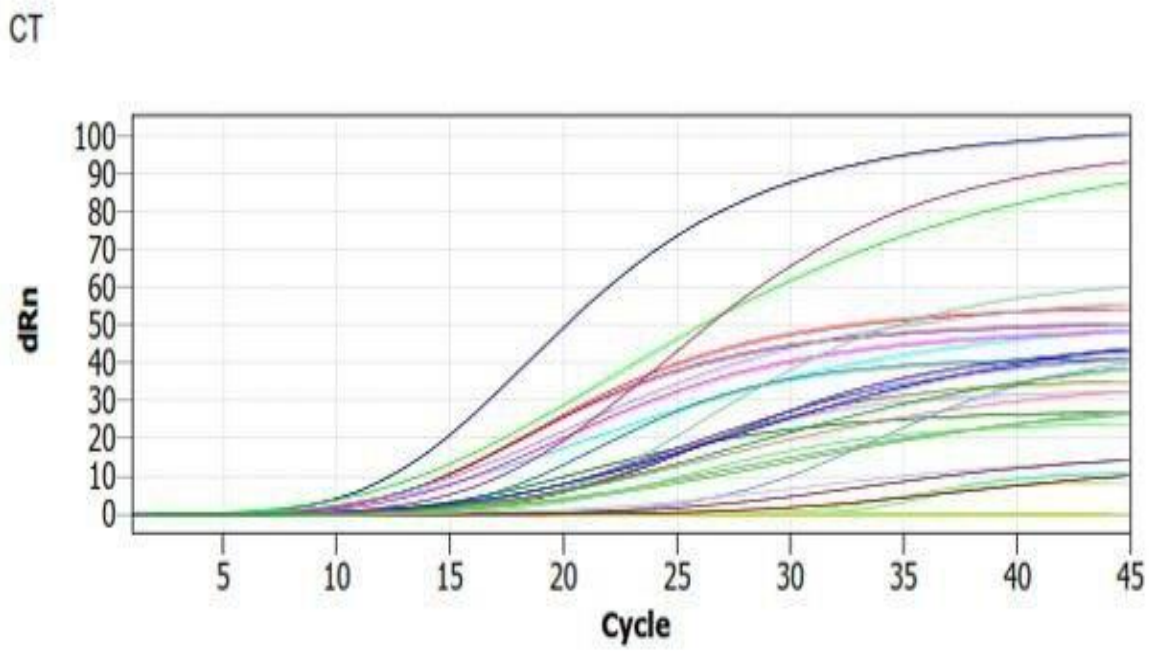
APPENDIX

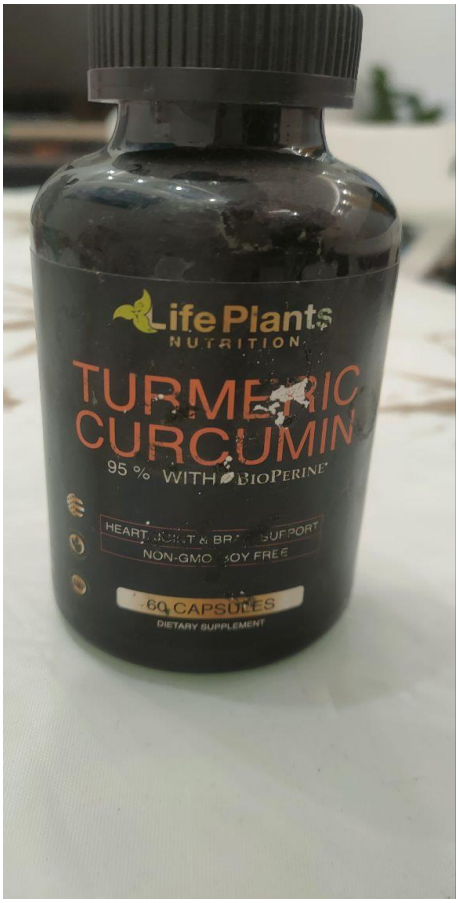
Appendix 1:- The Amplification Plots of *aph(3)-IIIa* , *aac(6')/aph(2'')* and *mecA* genes after and before the plant extracts

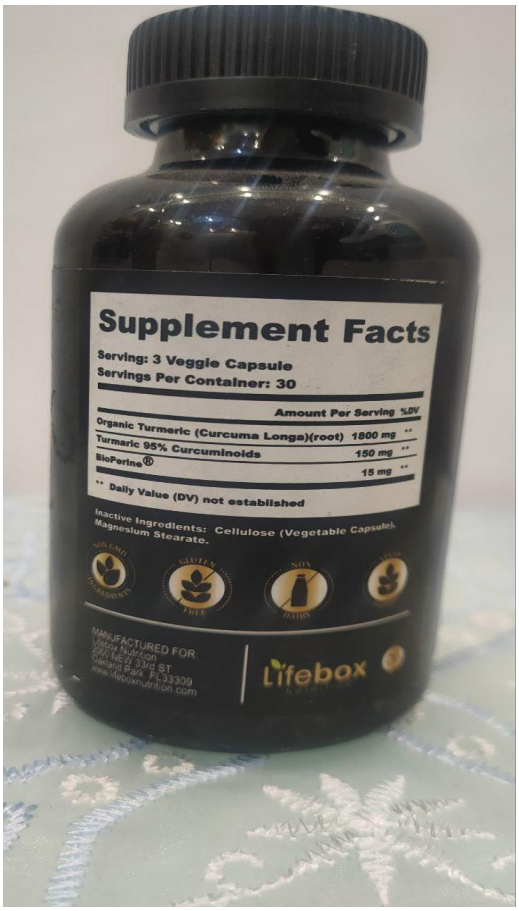




APPENDIX

Appendix 2 :- Curcumin extract packet





APPENDIX

Appendix 3 :- Berberine extract packet