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Evaluation of the effects of gold nanoparticles and *Tribulus terrestris* fruits extract on *atl*A gene expression in Methecillin Resistant *Staphylococcus aureus*

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Abstract

A total number of 10 methicillin resistant *Staphylococcus aureus* MRSA were collected from wounds, boils, and urinary tract infections at Al-Ramadi Teaching Hospital. Gold nanoparticles showed an inhibitory effect against all isolates at concentrations of 74.87, 56.15, 37.43, and 18.71 µg/ml with inhibition zones of 38, 26.7, 18.4, and 9.2 mm respectively, also ethanolic extract of *Tribulus terrestris* fruits showed an inhibitory effect against all isolates at concentrations of 100, 50, 25, and 12.5 µg/ml with inhibition zones of 25.6, 21.3, 16.6, and 11.3 mm respectively. Phytochemical screening showed eighteen bioactive compounds present in the ethanolic extract of *Tribulus terrestris* were identified by GC-MS analysis, the most important compounds phytol, Tetroxane, Pyrrole, Taraxerone, Lupeol, Linolic, Stearic, Oleic, which possess antibacterial properties. The results of gene expression showed *S.aureus* treated with Sub-MIC of AuNPs decreased atlA gene expression to (0.65). While the combination between AuNPs and extract was very effective against atlA gene expression to (0.05), but treated with extract alone increased gene expression to (1.77) compared with gene expression (1) of control group (bacteria without treatment).

Key words: Gold Nanoparticles, *Tribulus terrestris*, *Staphylococcus aureus* Methicillin Resistance, *atlA*, TEM.

INTRODUCTION

Staphylococcus aureus belongs to Micrococcaceae family and is part of the genus Staphylococcus, which contains more than 30 species such as *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*, but S. aureus is most virulent and pathogenic for humans. S. aureus is Gram-positive cell that may be observed as single cells, in pairs or as grape-like irregular clusters under microscope. It is characterized coagulase and catalase positive, non-spore-forming, non-motile, and facultative anaerobic **(1) (2)**. *Staphylococcus aureus* was a medical hazard 100 years ago, causing epidemics and fatal deaths from pneumonia, brain abscesses, meninges, septicemia and other deadly diseases **(3)**. Methicillin-resistant *Staphyloccoccus aureus* (MRSA) was first reported in 1961, within a year of methicillin introduction. Since then, MRSA strains have spread among hospitals and disseminated worldwide **(4)**. Autolysin gene is a newly discovered in *Staphylococcus aureus*. The gene product, atlA, is a unique, bi-functional protein that has an amidase domain and a glucosaminidase domain. It undergoes proteolytic processing to generate two extracellular peptidoglycan hydrolases, a 59-kDa endo-beta-N-acetylglucosaminidase and a 62-kDa N-acetylmuramyl-L-alanine amidase. It has been suggested that these enzymes are involved in the separation of daughter cells after cell division **(5)**. Autolysin gene is responsible for the production of enzymes that perform vital important functions, also contributes to the growth and division of bacterial cells and plays a major role in the initial stages of biofilm formation. The gene responsible for cell dispersion and formation of the cluster form **(6)**.

Nanomaterial's have the potential in the solution of many biological problems and the interference of nanotechnology with biology crystallizing the science of Nano-biotechnology **(7)**, it has been observed in recent years the rapid growth and advancement of nanotechnology and most of its applications in medicine. This improves the quality of life and the elimination of many of the problems we face in our lives **(8)**.

Tribulus terrestris L. is an annual plant commonly known as Puncture vine, Goat head, Devil's thorn and Gokhru. It is a flowering plant of the Zygophyllaceae family and has small yellow flowers containing only 5 petals, native to warm temperature and tropical region of the old world in Asia, Europe, Africa America, and Austraila **(9)**. The antimicrobial effects of *T. terrestris* reported in several studies, it is contains steroids, saponins, flavonoids, alkaloids, unsaturated and saturated fatty acids, vitamins, tannins, resins **(10)**.

The development and spread of antimicrobial resistance, as well as the emergence of new strains of pathogens, is a major concern for researchers, doctors, society and public health, therefore in this present research gold nanoparticles and *Tribulus terrestris* fruits extract investigated their effect on growth and gene expression of *atl*A gene in methicillin resistance *Staphylococcus aureus*.

MATERIALS AND METHODS

Chemicals and cultures

In the present study the chemicals used are Gold chloride (chloroauric acid; $HAuCl_4.4H_2O$), tri sodium citrate, CHROMagar, tryptone soya broth and mannitol salt agar, blood agar purchased from Oxoid Ltd., England, and sterile deionized water was used in this experiment.

Collection of clinical specimens

Samples were collected from the patients in the Ramadi educational hospital. Samples were cultured on the blood and MacConkey agar and incubated at 37 C° for 18-24 hours. After growth, the samples were kept at 4 C° until use. Then conducted Biochemical tests: In order to diagnose bacterial isolates, a number of biochemical tests were carried out in the approved diagnostic sources **(11)**, **(12)**.

Preparation of gold-nanoparticles (AuNPs) solution:

The method in **(13)** was adopted to prepare the solution of the AuNPs at a concentration of 74.87 µg/ml and the concentrations were obtained (56.15, 37.43, 18.71, 9.35) µg/ml as follow: Firstly, a $HAuCl_4.4H_2O$ solution with the concentration of 0.49 mol/L was prepared by dissolving 605 mg of $HAuCl_4.4H_2O$ into 3 ml of 10% HCl, then, a diluted 0.2 mM of $HAuCl_4.4H_2O$ solution was made by adding 40 µL (19.6 µmol) of $HAuCl_4.4H_2O$ solution into 100 ml of deionized water as to produce solution A. Secondly, 559 mg of Trisodium Citrate was added into 50 ml of deionized water to make solution B. The concentration of the solution was controlled at 38.8 mmol/L. Solution A was brought to a rolling boil at 150 C° with stirring vigorously as to get a homogenous size of the AuNPs solution. 10 mL of 38.8 mmol/L of sodium citrate was added rapidly into the vortex of the solution. The solution resulted in a color change from pale yellow to red. Boiling and stirring was continued for another 10 min. The heating was then removed, and stirring was continued for an additional 15 min. When the solution cooled down to room temperature, it was filtered through a 0.8 µm membrane filter paper. The prepared solution was kept in the refrigerator with the

temperature 4ºC and measured by using UV-Vis at the wavelength 400-800 nm, TEM and XRD.

Preparation of *Tribulus terrestris* ethanolic extract:

Powder sample (100 g) from plant fruit was extracted with ethanol by using a soxhlet apparatus for continuously 10 h or until the used solvent turned pure and colorless **(14)**. The solvent was removed by using a rotary vacuum evaporator at 40 °C to give a concentrated extract, and then powder-dried frozen until use.

Identification of phytocompounds by GC-MS analysis

The GC–MS analysis was carried out using a Shimadzu GC-MS-QP2010Ultra. The extract contains both polar and non-polar components of the material and 2ul sample of the solution was employed in GC-MS for analysis of different compounds. Interpretation on mass-spectrum GC-MS was conducted using the database of National institute Standard and Technology (NIST) having more 65,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were checked.

Antibacterial activity of GNPs and *Tribulus terrestris* ethanolic fruit extract against MRSA and determination of MIC

The antibacterial activity of synthesized GNPs and *Tribulus terrestris* ethanolic fruits extract were evaluated using disk diffusion method proposed by **(15)**. Pure cultures of selected bacteria were sub-cultured individually in tryptone soya broth for 18 hours at 37 C°. A 20 ml volume of sterile Mueller Hinton Agar medium was poured into each petri-plate and each isolate was swabbed uniformly into plates using sterile cotton swabs. Paper Disks from Whatman No.3 prepared by piercer means of a 6 mm diameter paper, sterilized with autoclave and impregnated with GNPs and extract for one hour, then left to dry thoroughly. Disks placed onto each bacterium inoculated agar plate by using sterile forceps. The bactericidal activity was determined by a clear inhibition zone around the sample loaded well after incubation of plates overnight at 37 C°. The minimum inhibitory concentration of GNPs and extract were determined by the method of **(16)** by preparing serial concentrations of GNPs (74.87, 56.15, 37.43, 18.71, and 9.35) µg/ml and *Tribulus terrestris* (100, 50, 25, 12.5, and 6.25) mg/ml. The lowest concentration inhibits the growth of bacteria considered MIC.

Gene Expression:

RNA extraction using Accuzol Reagent:

RNA was extracted and purified using quantification RNA extraction kit according to the manufacturer's instructions (Bioneer).

Real-Time PCR Amplification (One-step RT-qPCR):

The *atlA* gene was amplified using the forward primer (5'-TCACGCAATGGTTGTAGCTG-3') and reverse primer (5'-TTCGGGTTACCTCTTGCCAT-3') and primer of housekeeping *gyrB*-F (5'-CCAGGTAAATTAGCCGATTGC-3') and *gyrB*-R (5'-AAATCGCCTGCGTTCTAGAG-3') **(17)**. qPCR reaction was carried out in a 10ul reaction containing 5ul of qPCR Master Mix (Promega, US), 0.5 ul of 10 pmol/ul of each primer and Prob 5'Fam ACCAACAGCCAACAATGGTTCGGCA-3'Tamra (Macrogen, South Korea), 1.5ul of RNA template,0.25 of RT(Reverse Transcriptase) enzyme (Promega, US) and the volume was completed to 10ul using nuclease-free water. Thermo cycling conditions were as follows:35oC for 5min., 42oC for 15min.,95oC for 10min.(1cycle), followed by 45 cycles of denaturation at 95oC for 30sec; annealing at 60oC for 30sec; extension at 72oC for 30sec., followed by a melting profile: Collect data starting at 72°C to 95oC at 0.3oC/s

Calculated of Ct value depend on Livak equation as follow:

ΔCt(treated sample) = CtGen – Ct gyrB

ΔCt(control) = CtGen – Ct gyrB

$\Delta\Delta Ct = \Delta Ct$(treat.) - ΔCt(con.).

Folding = $2^{-\Delta\Delta Ct}$.

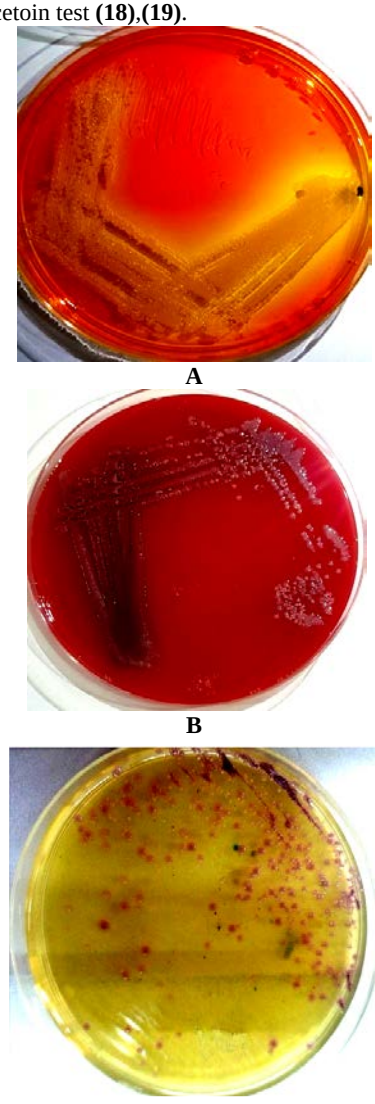
Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and was analyzed using Two-way ANOVA at 5% level of significance using computer software SPSS version 22.

RESULTS AND DISCUSSION

Isolation and Identification of *Staphylococcus aureus* isolates:

Among 83 clinical samples, 10 isolates were found to be Methicillin Resistance *Staphylococcus aureus*; The microscopic examination showed gram positive cocci arranged as clusters and characterized as positive for catalase, negative for oxidase test, and fermentation of glucose, and that appeared circular white to creamy colored with smooth edges and slightly higher on the surface of blood agar. They were characterized as purple color in MRSA CHROMagar medium that selective medium for MRSA. *Staphylococcus aureus* was distinguished from other species belonging to Staphylococcus genus depends on mannitol salt medium and its fermentation of mannitol sugar by converting the color of medium from red to yellow Figure (1), as well as its ability to produce coagulase, DNase, hemolysin enzymes and positive to acetoin test **(18)**,**(19)**.



A

B

C

Figure (1): A- Growth of MRSA on CHROMagar, B- Growth of MRSA on Blood agar, C- Growth of MRSA on Mannitol salt medium

Synthesis of Gold Nanoparticles

Synthesis of gold nanoparticles was observed through a number of indicators as follows:

Color change: The results of present study showed that the gold nanoparticles (AuNPs) formation by citrate reduction of the gold chloride solution, and appearance of red grape wine color (Figure 2). This chromatic change is caused by irritation of plasmon surface nanoparticles **(20)**.

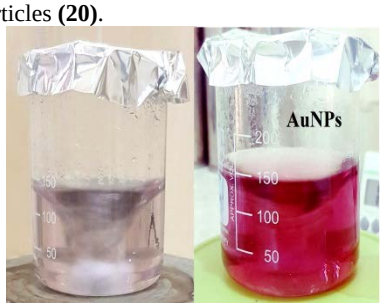


Figure (2): Color change of gold chloride solution and AuNPs Synthesis

Absorption UV- light spectroscopy: The results of present study showed the production of AuNPs by measuring the absorption spectra of UV-visible within the range (400-800) nm for gold chloride solution that used to prepare nanoparticles, which are important techniques for the detection of nanoparticles, the peak of absorption appeared at wavelength 520 nm (Figure 3), which represents the peak absorption of gold that agree with **(21)**, **(22)**.

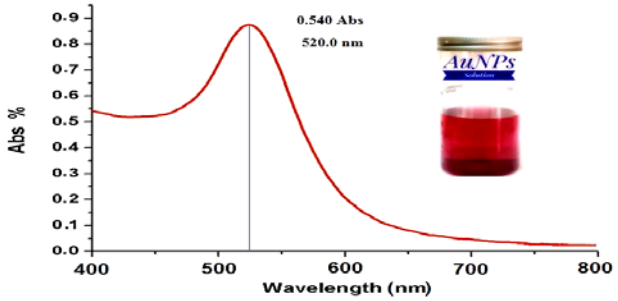


Figure (3): UV- visible light absorption of AuNPs solution

X-Ray Diffraction: Figure (4) shows the X-ray diffraction spectra of the gold nanoparticles and observe the peaks of diffraction (111), (200), (220), (311) and (222) at angles (38.2 °, 44.4 °, 64.6 and 77.6 and 81.7 respectively). These angles were found to be close to the angles indicated with the JCPDS card and agree with **(23)**, **(24)**.

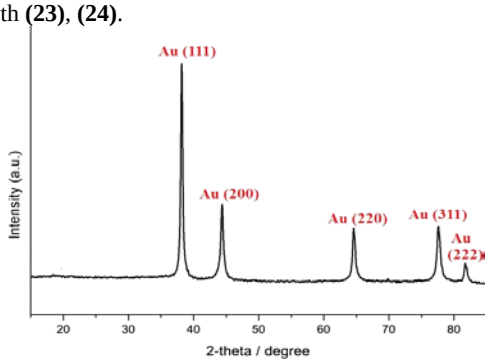


Figure (4): X-ray diffraction spectra of AuNPs

Transmission Electron Microscope (TEM): Figure (5) shows the shape of the gold nanoparticles in TEM with a magnification force of 46000 X, range of diameter between (17-25) nm. Particles are shown in clusters which confirm that nanoparticles are formed **(25)**.

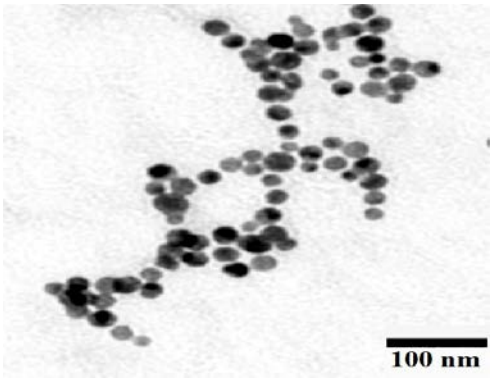


Figure (5): shape of gold nanoparticles in TEM with 46000 X magnification force

Antibacterial activity of AuNPs against MRSA

The results showed that AuNPs were highly effective against MRSA as shown in table (1), and figure (6) that show the diameter average of the inhibition zones (38, 26.7, 18.4, 9.2 and 0) mm at concentrations (74.87, 56.15, 37.43, 18.71, and 9.35) µg/ml respectively.

Table (1): Effect of AuNPs with different concentrations in MRSA growth

	AuNPs Concentrations µg/ml	Inhibition zone in mm (Mean ± SE)
1	**74.87**	**38** ± 1.7
2	**56.15**	**26.7** ± 1.3
3	**37.43**	**18.4** ± 0.9
4	**18.71**	**9.2** ± 0.6
5	**9.35**	**0** ± 0.0

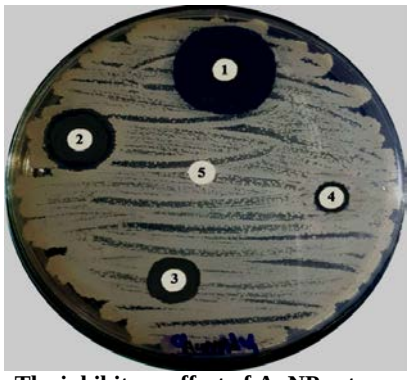


Figure (6): The inhibitory effect of AuNPs at concentrations (74.87, 56.15, 37.43, 18.71, and 9.35) µg/ml against MRSA isolates

The small size of AuNPs and their large surface area play important role in the toxicity. Whenever smaller size, accumulation is greater on the surface of the cells, which increases the toxicity against the bacteria through its effect on plasma membrane permeability leading to the death of bacterial cell **(26)**. The mechanism of nanoparticles action that interacts with bacterial cells, the bacterial cells have negative charges while the metal oxides have a positive charge, which creates electromagnetic attraction between the bacteria and particle surfaces. The particles release the ions that interact with thiol (-SH) group of transport proteins that emerge from the membrane of the bacterial cell and reduce the permeability of the membrane leading to bacterial cell death **(27)**. Gold nanoparticles attack surface of the cell membrane, disrupt permeability and cellular respiration functions or interfere with system components of electron transport chain in bacteria **(28)**.

Table (2): GC–MS analysis of *Tribulus terrestris*

No.	R.T	Name (IUPAC)	Chemical Formula	M.W g/mol	Peak Area%
1	6.09	1,3,5,7-Tetroxane	$C_4H_8O_4$	120.104	1.17
2	7.34	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.126	1.47
3	13.61	1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)	$C_6H_{12}O_4$	148.158	0.37
4	16.05	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194.183	8.50
5	16.67	Phytol;3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.539	0.12
6	17.37	Palmitic acid; Methyl Palmitate; Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.457	0.15
7	17.56	Pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	210.27	0.03
8	17.73	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.403	24.77
9	18.73	Taraxerone	$C_{30}H_{48}O$	424.713	1.22
10	18.82	Linolic acid ;9,12-Octadecadienoic acid (z,z)-	$C_{21}H_{40}O_2Si$	352.634	0.39
11	19.16	Stearic acid; Octadecanoic acid	$C_{18}H_{36}O_2$	284.484	20.29
12	20.11	Lupeol; Fagarasterol; Clerodol	$C_{30}H_{50}O$	426.729	3.12
13	20.60	Lupeol acetate; Lup-20(29)-en-3-ol, acetate, (3.beta.)-	$C_{32}H_{52}O_2$	468.766	27.02
14	21.43	2-Palmitoylglycerol; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330.509	0.22
15	21.73	Diisooctyl phthalate; 1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390.564	0.16
16	23.62	Erucamide13-Docosenamide, (Z)	$C_{22}H_{43}NO$	337.592	0.14
17	23.89	Methyl Elaidate; Elaidic acid methyl ester	$C_{19}H_{36}O_2$	424.713	4.44
18	24.36	Oleic acid; Cis-9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.468	6.42

GC-MS Analysis of *Tribulus terrestris* extract

Eighteen bioactive compounds present in the ethanolic extract of *Tribulus terrestris* were identified by GC-MS analysis table (2). Spectrogram shows the peak identities of the compounds. Active principles with their Retention Time (RT), Chemical Formula (MF), Molecular Weight (MW), peak area (%) are presented in Table (2). Eighteen chemical constituents have been identified; the major chemical constituents are Tetroxane, Phytol, Pyrrole, Taraxerone, Lupeol, Linolic, Stearic, and Oleic (Table2&Fig7).

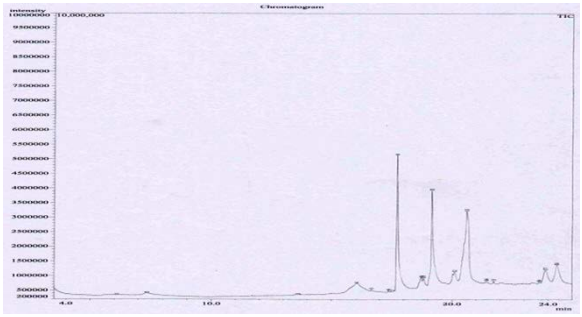


Figure (7): GC–MS analysis of *Tribulus terrestris*

Antibacterial activity of *Tribulus terrestris* ethanolic extract against MRSA

The extract of fruits of *Tribulus terrestris* was showed considerably good antibacterial activity with diameters of zone of inhibition average between 11.3-25.6 mm (table3). MIC value of ethanolic extract of fruits was 12.5 mg/ml against MRSA isolates (table3 & fig 8).

Table (3): Effect of *Tribulus terrestris* extract with different concentrations in MRSA growth

T. terrestris Concentrations(mg/ml)	Inhibition zone in mm(Mean ± SE)
100	25.6 ± 1.3
50	21.3 ± 1.1
25	16.6 ± 0.3
12.5	11.3 ± 0.7
6.25	0 ± 0.0

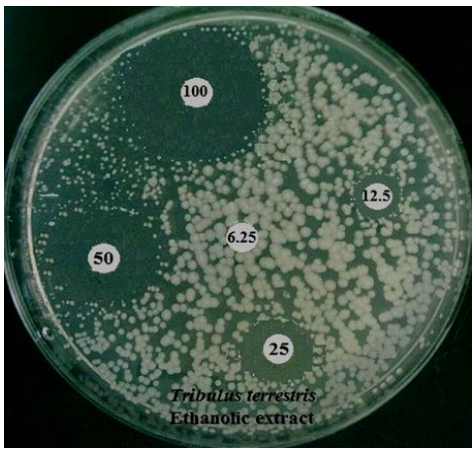


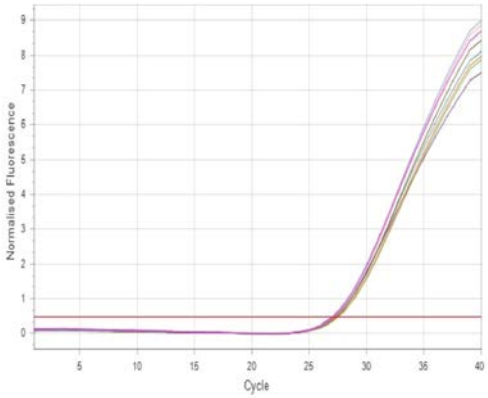
Figure (8): The inhibitory effect of *T. terrestris* at concentrations (100, 50, 25, 12.5, and 6.25) mg/ml against MRSA isolates

The investigation of phytochemical analysis of ethanolic extract of *T. terrestris* fruits showed the presence several bioactive compounds, which are the main reason for antibacterial activity of extract. The most bioactive compounds we have obtained from GC-MS analysis were phytol which most studies refer to its role in antimicrobial killing; this is confirmed by **(29)** the phytol has antimicrobial activity against *S.aureus, E.coli, C. albicans, Aspergillus niger*. 'Phytol induced Reactive Oxygen Species accumulation and that the electron transport chain was involved in increase of ROS. Due to this ROS generation, the imbalance developed between intracellular ROS and the antioxidant defense system, leading to decrease of reduced glutathione (GSH). Moreover, severe DNA damage was shown after treatment with phytol' **(30)**. The containment of the plant extract on the fatty acids saturated Palmitic, Stearic acid, and unsaturated Linoleic, Oleic acid, enhances the inhibitory effectiveness of bacterial growth **(31)**. The plant extract was characterized by its containment of Pyrrole fatty acid, which possess and its derivatives inhibitory and killer properties for Gram positive and negative bacteria and fungus **(32)**. The presence of flavonoids (Lupeol, Lupeol acetate) enhances the antibacterial activity of

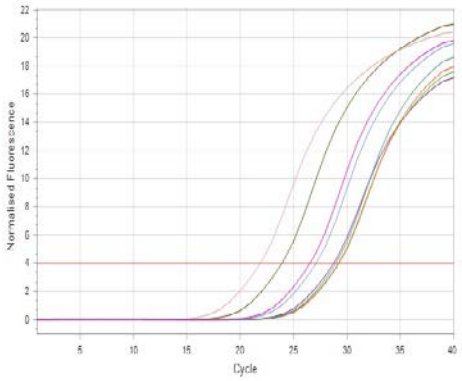
plant extract, the mechanisms of these compounds blocking of certain enzymes that play important role in growth, reproduction, cell rupture or fuctional change in bacteria **(33)**.

Effect of AuNPs, *Tribulus terrestris* extract, and combination between them on *atlA* gene expression

RNA was extracted for samples that treated with Sub-MIC of AuNPs, *T. terrestris* extract, and combination between them. RNA was obtained at high concentrations, ranging between 167-518 ng/µl. Cycle Threshold (Ct) considers the basic rule that expresses about the amount of gene expression in qRT-PCR figure (9).



A



B

Figure (9): A- Curves of Ct values of *atlA* gene, B- Curves of Ct values of *gyrB* housekeeping gene of MRSA *S.aureus*

The results showed a significant effect at P <0.001 for groups that treated with Sub-MIC of AuNPs and AuNPs combined with *T. terrestris* extract, gold-nanoparticles combined with extract reduce amount of *atlA* gene expression to a large extent, where the gene expression was (0.05). Whereas reported (0.65) for group that treated with AuNPs alone. As for control group without treatment was (1). In contrast, the results showed a significant increase of *atlA* gene expression for group that treated with Sub-MIC of extract alone, where it became (1.77) (table 4 & figure 10).

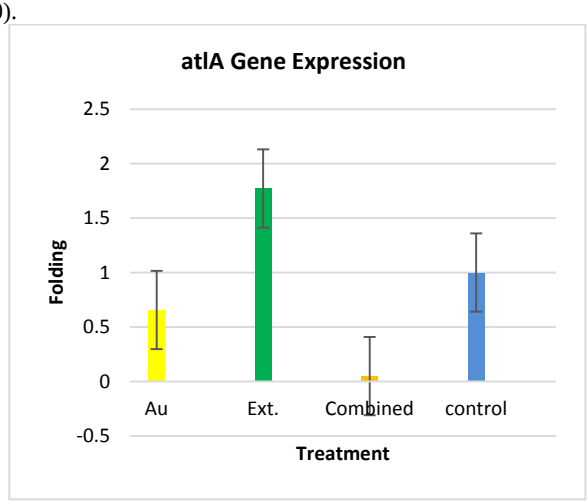


Figure (10): Effect of AuNPs, Extract, and combination between them with Sub-MIC on *atlA* gene expression

The results showed that Sub-MIC of AuNPs reduced *atlA* gene expression in the MRSA *S.aureus* bacteria. This is due to the effect of particles on protein functions and the destruction of DNA, AuNPs Inhibitor of bacterial growth and the effect on proteins folding to missing function **(34)**. The molecular mechanism of AuNPs is interfering with the process of bacterial protein synthesis. These particles block the link of the ribosome subunit with the tRNA, disrupt the cell membrane potential, inhibit ATPase activity and reduce the energy level **(35)**. The combination between AuNPs and T.T extract in equal quantities affected significantly in *atlA* gene expression reduction that means the ability of AuNPs to binding with bacterial cell membrane and interfere with membrane lipids and penetrate into the bacterial cell, also AuNPs have the characteristic of drug-delivery. AuNPs are loaded with plant extracts molecules in addition to the holes produced at the wall of the bacterial cell and the arrival of the extract into the cell to work inhibit the receptors and interference with the process of gene expression and the production of inactive enzymes **(36)**. Plant extract alone was unable to penetrate of bacterial cell membranes and remained at surrounding area, thus bacteria adapting against this concentration, for this reason the measuring of gene expression very important to determine how adaptation occur with different environmental conditions **(37)**, this concentration stimulated bacterial resistance and considered it an exogenous effect representing the increased amount of gene expression.

Table (4): Values of *atlA* and *gyrB* gene expression

Group	Sample	*gyrB*	*atlA*	DCt		DDCt	Folding
Au	**A1**	26.73215	27.12524	0.393086	0.750241	0.608641	**0.655814**
	A2	26.42641	27.53381	1.107396			
Ext.	**T1**	28.18141	27.42989	-0.75152	-0.68384	-0.82544	**1.772078**
	T2	27.89192	27.27576	-0.61616			
Combined	**Co1**	23.83623	27.31984	3.483602	4.458298	4.316698	**0.050182**
	Co2	21.81043	27.24342	5.432994			
control	**C1**	27.03975	27.14054	0.100788	0.141601	0	**1**
	C2	26.85263	27.03504	0.182415			

CONCLUSIONS

Based on the results, it can be concluded that CHROMagar medium very important in diagnosis of *Staphylococcus aureus* methicillin resistant. Gold-Nanoparticles and ***Tribulus terrestris* fruits ethanolic extract** have Strong efficacy against bacterial, combination between them give best result against bacterial inhibition, prevent of biofilm production, decreasing of atlA gene expression compared with using of extract alone, because AuNPs work as drug delivery and possess ability to penetrate of bacterial membranes.

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