Original Article

Gene expression study of Pvl genes in Methicillin-Resistant Staphylococcus aureus bacteria on the effect of extract Tribulus terrestris L.

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Abstract

125 samples were collected from different clinical sources including (burns, wounds, ear, suppurations and boils, urine) from Ramadi Teaching Hospital and Women's and Children's Hospital in Ramadi for the period from (11-2021 to 1-2022). 50 isolates of Staphylococcus aureus were found, of which 26 were MRSA isolates, with an isolate rate of (52%). The ethanolic extract of the fruits of the Tribulus terrestris Lplant was prepared and its effectiveness against the isolates was tested by diffusion around the pits. The ethanolic extract had anti-MRSA activity by8 5%. The general average diameter of the inhibition for isolates was 18.038 mm, and the general average for MIC was 14.76 mg/ml. The results of GC-MS showed that the extract contained 49 effective chemical compounds, the most important of which are phytol, pyrrole, saturated and unsaturated fatty acids, alkaloids, saponins, tannins and phenols. The gene expression results showed that the MRSA bacteria treated with sub-MIC for the plant extract reduced the gene expression of PVL genes (lukS-pv and lukF-pv) to 0.506,0.716 respectively compared to the gene expression (1) of the control group (untreated bacteria). It was found that the ethanolic extract of the plant is able to degrade red blood cells at a concentration of 750 mg/ml.

Keywords: Staphylococcus aureus Methicillin Resistance, Tribulus terrestrisL , PVL(lukS-pv and lukF-pv), Gene expression, GC-MS

Introduction

The bacteria Staphylococcus aureus was discovered in the year 1880 in Aberdeen - Scotland by the surgeon Auguston, by isolating this bacteria from pus, then the scientist Frederick Jules came to name this bacteria later (Frank et al.,2004). Staphylococcus aureus includes the genus methicillin-resistant Staphylococcus aureus (MRSA), which is found naturally in both the community and healthcare facilities, and is associated with high morbidity and mortality and has high medical costs (Purrello et al.,2016). The increasing prevalence of MRSA infection worldwide has led to health concerns and can occur in healthy individuals who lack access to health care(Aqel et al.,2015).

PVL toxin is prevalent in Staphylococcus aureus in general by 5%, and it is the gene that produces a series of chemicals that make the MRSA bacteria have the ability to resist, invade and cause diseases in children and young adults (David and Daum,2010). PVL-cell toxin encodes for two exoprotein subunits(lukS-pv and lukF-pv) (D.C.Melles et al.,2006). They have a role in the breakdown of white blood cells and myeloid cells (Graves et al.,2010).

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Tribulus terrestris L It germinates in April and blooms and remains green throughout the summer and then dries up and dies (Suresh et al.,2016). It spreads naturally in the Iraqi forests of Mosul, Habbaniyah, Al-Rutbah, Sulaymaniyah and the Najaf desert (Qasem,2014). Tribulus terrestrisL is used as a diuretic, antioxidant, weight reducer and stroke treatment(Jiang YH et al.,2017) It is used as an antibacterial (Chhatre et al.,2014)

The plant contains many effective compounds, including steroidal saponins, alkaloids, and flavonoids (Semerdijieva and Zheljazkov,2019)It is also extremely rich in unsaturated fats, acids, vitamins, and tannins (Khazaei et al.,2018).The aim of our study was to stud the gene expression of PVL (lukS-pv and lukF-pv) genes in methicillin-resistant Staphylococcus aureus (MRSA) upon the effect of Tribulus terrestris L extract.

Classification of the Tribulus terrestris L
Class: Dicolyledons
Sub class:Polypetatae
Series:Thalamiflorae
Order:Geraniales
Family:Zygophyllaceae
Genus:Tribulus
Species:terrestris
(Jagadeesan et al.,2005)

Materials And Methods

Collection and identification of isolates

125 samples were collected from different clinical sources including (burns, wounds, ear, suppurations and boils, urine) from patients of Ramadi Teaching Hospital and Women and Children Hospital in Ramadi city for the period from month (11-2021 to 1-2022). Then they were cultured on blood agar, McConkey agar and mannitol salt agar, and incubated at 37C for 24 hours, and upon growth, the samples were kept at 4 Cdegree until use, then biochemical and molecular tests were performed on them

Preparation of the ethanolic extract of the fruits of the Tribulus terrestris L plant

The fruits were collected, washed well, and then left to dry at room temperature, and after confirming their air dryness, they were ground into a fine powder by an electric mill. The ethanolic extract of the fruits of the Tribulus terrestris L plant was prepared according to the method (Mohammed et al.,2010). Powder 50 g of the plant was prepared in 500 ml of 80% ethanol alcohol in a soxhlet machine for 6consecutive hours at a temperature of 75-80. Temperature 37 and after drying the extract was raised and then kept in glass tubes in the refrigerator until use, Figure (1),(2)

Figure 1: Tribulus terrestris L plant powder

Figure 2 : Tribulus terrestrisT plant extract in Soxhlet

Identification of chemical compounds in the extract of Tribulus terrestris L fruit using GC –MS technique:By means of a gas chromatograph connected to a mass spectrometerGC-MSQP2010Ultra(Shimadzu Japan), The active chemical compounds of the plant were identified.

Anti-bacterial activity test of extract of Tribulus terrestris L fruit:The method was relied on (Valgas et al.,2007), which is the method of spreading around the pits, pits are formed on the center of Muller-hanton agar, and then the bacterial suspension is spread, and the dishes are left for 5minutes to absorb the suspension, The extract was placed inside the pits, and the dishes were incubated for 24 hours at a temperature of 37, after which the diameter of the damping area (mm) around the pits of the extract was calculated.Figure (3)

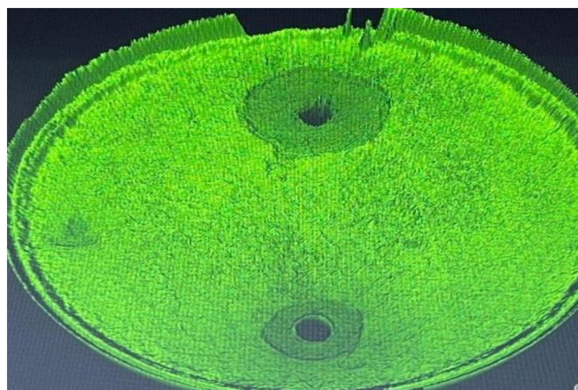

Figure 3: Inhibitory activity of plant extract against bacterial isolates

Determine the minimum inhibitory concentration (MIC) of the extract:Depending on (Arendrup et al.,2017) determine the minimum inhibitory concentration of Tribulus terrestris L fruit extract against all isolates of methicillin-resistant Staphylococcus aureus(MRSA).

Cytotoxicity test of ethanolic extract of Tribulus terrestris L fruit

The toxicological activity was determined based on the method (Xin-guo and Ursella,1994) by making concentrations of the extract (125,250,500,750) mg / ml, 0.8 ml of each concentration was taken and placed in a sterile test tube, and 0.2 ml human red blood cell was added, so that the final volume was 1 ml. The tubes were kept in the incubator for half an hour at 37°C, then centrifuged for 5 minutes, after which the hemolysis was compared with a test tube containing only blood (controll).

Gene Expression

A- RNA extraction: According to the manufacturer's instructions (Bioneer), the RNA was extracted, and then the acid concentrations were measured using a Quantus Fluorometer device.

B-Real-Time PCR(one-step qRT- PCR)

Replication steps were carried out to investigate the amount of gene expression of titration gene 16 and PVL genes (lukS-pv and lukF-pv) of untreated bacterial isolates treated with plant extract at the concentration under the minimum inhibitor Sub-MIC. Through the use of the special primers for each gene

(CGATTCCAGCTTCATGT/TGTCGTGAGATGTTGGG, TTCAGGGTTTTCAACAGTAGCA/ACACAATTGCCAG CGGTAAAA,GTTTTCGCCAGACCAATAGCC/ GCCTGTAACTGTGTCTGAAGG), The reaction components were prepared by mixing 10 µl of master Mix ,5µl ofcDNA,1 µl of Forward Primer,1 µl Reverse Primer and 3 µl Free Nuclease The final volume is 20 µl. Through the use of a kit supplied by Luna qPCR Biolabs (England)Kit company, Rely on the system of cycles of the interaction of the qRT- PCR table (1).

Table 1: Interaction cycle system qRT –PCR

Step	*Temperature*	*Time*	*Cycle*
primary Denaturation	95C˚	10 min	1
Denaturation	C˚95	Sec10	
Annealing	60C˚	Sec30	39
Extension	93C˚	5 Sec	
End extension	95 C˚	20 Sec	1

C- Quantification of gene expression (Ct) using the Livack equation

According to the following equation, gene expression is measured:

ΔCt (Treated sample) = CtGen – Ct H.K

ΔCt (Control) = CtGen – Ct H.K

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

Folding = 2-$\Delta\Delta$Ct

Statistical analysis

After collecting and tabulating the data related to the current study, they were statistically analyzed according to the Simple Experimental system applied using the Complete Randomized Design (CRD), and using the statistical program GenStat - Tenth Edition Version -10.3.0.0, and the significant differences of the averages were tested using a lower test. Significant L.S.D at 0.05 probability level, 0.01 probability level, and power P-value 0.05

Results And Discussion

Collection and identification of isolates

Among 125 clinical samples, 50 isolates of Staphylococcus aureus were found, 26 of them were methicillin-resistant Staphylococcus aureus (MRSA), with a percentage of 52%, and this percentage is in agreement with(LevaNam et al.,2019). The results of the culture examination showed that the colonies growing on the medium of the blood agar were surrounded by a clear area of beta-hemolysis, with a diameter (1.5-2.5)mm of white or golden round (mm). While on the mannitol salt agar medium, the medium-sized colonies with smooth, shiny edges and slightly raised from the center, surrounded by a yellow region that turns the color of the center from pink to yellow, the mannitol salt agar medium is one of the selective media for the genus Staphylococcus aureus(Mac Faddin,200). As for the microscopic examination, the isolates appeared in the form of grape clusters positive for the Gram stain(Matar,2014). Also, biochemical tests were performed on her, which were positive for catalase, negative for oxidase, fermentation of mannitol and negative for indole.

Identification of chemical compounds in the extract of Tribulus terrestris L fruit using GC –MS technique

GC-MS technology is an analytical technique that can be used to separate and analyze samples that are evaporated without thermal decomposition (Nokhala et al.,2020). 49 active chemical compounds were detected in the ethanolic extract of prickly pear fruits, which contains saponins, phytol, pyrrole, saturated and unsaturated fatty acids, alkaloids and aromatic compounds. Table (2) Figure (4).

Table 2: GC-MS analysis of Tribulus terrestris

No	RT (min)	Area%	Name	Quality	CAS Number
1	5.15	0.14	2(3H)-Furanone, 5-methyl-	80	000591-12-8
2	5.477	0.14	cis-2,5-dimethylpyrrolidine	64	039713-71-8
3	5.799	0.32	2-Ethylpiperidine	78	001484-80-6
4	6.588	1.15	Pentanal	9	000110-62-3
5	8.149	0.26	HYDROXY DIMETHYL FURANONE	74	003658-77-3
6	8.793	0.15	1,3-Dimethyl-2-thioketoimidazole	52	000000-00-0
7	8.928	1.24	Guaiacol	94	000090-05-1
8	9.483	0.44	Maltol	72	000118-71-8
9	10.82	0.74	Uracil, 1-methyl-4-thio-	27	035455-86-8
27	28.62	1.16	9-Octadecenoic acid (Z)-, methyl ester	99	000112-62-9
32	31.46	0.22	Cyclononanone	38	003350-30-9
33	32.29	0.18	Ethyl linoleate	60	000544-35-4
34	33.83	1.35	Bicyclo[10.1.0]tridec-1-ene	94	054766-91-5
35	34.31	1.04	Palmitic acid .beta.-monoglyceride	87	023470-00-0
36	36.18	0.17	Nitroscanate	60	019881-18-6
37	36.58	6.73	Ethyl linoleate	93	000544-35-4
38	36.89	0.25	12-Cyano-15-pentadecanolide	55	112405-38-6
39	41.59	0.72	Lanol	99	000057-88-5
40	41.76	0.31	Vitamin e	95	010191-41-0
41	43.09	0.51	Campesterol	97	000474-62-4
42	43.58	0.80	Stigmasterol	89	000083-48-7
43	44.02	1.51	Diosgenin	97	000512-04-9
44	44.24	0.31	Smilagenin	78	000000-00-0
45	44.54	3.80	(23S)-ethylcholest-5-en-3.beta.-ol	99	113845-28-6
46	44.83	0.31	23,24-BISNORCHOLA-5,17(20)-DIEN-3.BETA.-OL	64	072654-92-3
48	45.35	0.23	Tigogenone	52	000470-07-5
49	45.88	0.14	5-Acetamido-4,7-dioxo-4,7-dihydrobenzofurazan	41	000000-00-0

Figure 4: GC-MS analysis of Tribulus terrestris.

Anti-bacterial activity test of extract of Tribulus terrestris L fruit

Our study showed that the ethanolic extract of the fruits of the prickly pear plant had an antibacterial activity of MRSA by85%, the general average of the inhibition diameters of the isolates was 18.038 mm, table(3). While the overall mean value of MIC for the ethanolic extract of the plant was 14.76mg/ml towards MRS isolates, Table (4). The researcher found (Ammar et al.,2018) that the extract of the Tribulus terrestris L plant has aneffective activity against Escherichia coli,Staphylo coccusaureus and Salmonella bacteria because the plant contains phenols, flavonoids andsaponins that were detected by GC-Mtechnology. Also(Abdulqawi and Quadri,2021) it was found that the plant extract was antibacterial against MRSA bacteria due to its possession of alkaloids, tannins, glycosides, flavonoids, phenols and saponins. These studies were in agreement with our study. The presence of fatty acids in the plant helps to destroy the vital membranes as well as their resistance to the pumps efflex mechanical (Papaianni et al.,2020). It was found that the pyrrole compound has anti-bacterial activity against gram-positive and gram-negative bacteria as well as anti-fungal, which works to damage cell membranes and prevent the activity of the reverse transcriptase enzyme and protein enzymes of the cellular system (V.B handwaj et al.,2015).

Table 3: Diameters of inhibition rates for plant extract

No.	Isolates	T.T
1	S. aureus	18
2	S. aureus	22
3	S. aureus	19
4	S. aureus	16
5	S. aureus	20
6	S. aureus	16
7	S. aureus	21
8	S. aureus	22
9	S. aureus	22
10	S. aureus	17
11	S. aureus	18
12	S. aureus	14
13	S. aureus	14
14	S. aureus	16
15	S. aureus	15
16	S. aureus	18
17	S. aureus	16
18	S. aureus	20
19	S. aureus	18
20	S. aureus	16
21	S. aureus	16
22	S. aureus	18
23	S. aureus	19
24	S. aureus	18
25	S. aureus	18
26	S. aureus	22
Mean		18.038
S.E.		0.555
%C.V		5.326
L.S.D 5%		1.574
L.S.D 1%		2.097
P< 0.05 level		0.00012

Table 4: MIC plant extract account

No.	Isolates	T.T
1	S. aureus	16
2	S. aureus	8
3	S. aureus	8
4	S. aureus	16
5	S. aureus	16
6	S. aureus	8
7	S. aureus	16
8	S. aureus	8
9	S. aureus	16
10	S. aureus	16
11	S. aureus	16
12	S. aureus	8
13	S. aureus	16
14	S. aureus	32
15	S. aureus	32
16	S. aureus	8
17	S. aureus	16
18	S. aureus	8
19	S. aureus	16
20	S. aureus	16
21	S. aureus	32
22	S. aureus	16
23	S. aureus	16
24	S. aureus	8
25	S. aureus	8
26	S. aureus	8
Mean		14.76
S.E.		0.299
%C.V		3.513
L.S.D 5%		0.850
L.S.D 1%		1.132
P< 0.05 level		0.0011

Cytotoxicity test of ethanolic extract of Tribulus terrestris L fruit

The result of the toxic activity was shown at a concentration of 750 mg / ml by destroying red blood cells figure(5), our results were in agreement with (Ivanova et al., 2016), where it was found that barbed pole extracts are toxic to cells because they contain saponins. Saponins are able to break down red blood cells and cause anemia (Hu et al., 2018). This is due to the affinity of saponins to the sterols involved in the composition of the plasma cell membrane, by removing the membrane and releasing hemoglobin in the blood (Arslan and Celik, 2013).Also, our current study agreed with the researcher (Ghanem, 2016) who found that the extract of this plant is cytotoxic and antimicrobial, so it is recommended to use the pharmaceutical method for biologically active compounds after conducting toxicological activity tests for the plant.

Figure 5: Cytotoxic efficacy test of the Tribulus terrestris L plant

Effect of plant extract (Tribulus terrestris L) on gene expression of PVL(lukS-pv and lukF-pv)genes

The RNA of the first sample of bacteria treated withSub- MI Cwas extracted from the Tribulus terrestris L extract, as well as the RNA extraction of the same sample without treatment.(Control). The RNA concentration of the treated sample was(176 ng/microliter), and the RNA concentration of the untreated sample was (234 ng/microliter). Ct is the basic rule by which the amount of gene expression can be expressed in -qRTPCR technique Table (5and 6).

Table 5: Effect of Tribulus terrestris L extract on the amount of gene expression of lukS-pvl gene at sub-MIC concentrations.

Groups	2(-ΔΔCt)	Folding	Conclutions
Tribulus terrestris L	0.506	1.97628	Down-regulation
Control (without)	1		

Table 6: Effect of Tribulus terrestris L extract on the amount of gene expression of lukF-pvl gene at sub-MIC concentrations.

Groups	2(-ΔΔCt)	Folding	Conclutions
Tribulus terrestris L	0.716	1.396648	Down-regulation
Control (without)	1		

Our study showed that the Tribulus terrestris L plant reduced the amount of gene expression of PVL(lukS-pv and lukF-pv) genes by a value of (0.506,0.716) respectively compared to the control group that was not treated with a value of (1). The plant extract helps prevent biofilm production, which leads to a reduction in the amount of gene expression through the plant's ability to penetrate the membranes of bacteria(AL-Taee et al.,2018). The ethanolic extract has the ability to create holes in the bacterial cell wall, causing chaos to the bacteria(Mushtag et al.,2018). The plant's possession of many effective chemical compounds helps it to denature the bacterial protein attached to the bacterial cell wall and cause it to decompose because it contains flavonoids(Chauhan and Goel,2018).

Conclusions

The extract of Tribulus terrestris L fruit is considered to have an antagonistic activity against MRSA bacteria, as it reduces the gene expression of PVL (lukS-pv and lukF-PV) genes by a value of (0.506,0.716) respectively. It also has the ability to degrade red blood cells at a concentration of 750 mg / ml

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