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STUDY THE EFFECT OF *ROSMARINUS OFFICINALIS L.* ESSENTIAL OIL ON TYPE1&TYPE3 FIMBRIAE GENES IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIAE*

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Abstract

Klebsiella pneumoniae bacteria are opportunistic pathogen.This bacterium have the ability to produce biofilm which is a virulence factor .The aim of current project was to study the effect of rosemary essential oil on inhibition of biofilm in (Strong biofilm producer and multi drug resistance isolates) and also to evaluate the action of this oil in MICs concentrations in gene expression of (*fimh* and *mrkd* genes) for type 1 and type 3 fimbriae respectivelly against three isolates .the results of antibiofilm activity showed that there were a significant differences ($P \leq 0.05$) in OD values of biofilm formation between *K. pneumoniae* isolates before and after treatment with Rosemary oil, and the biofilm inhibition % was also determined.The results of gene expression pre and post treatment with 1/5 MICs value of rosemary oil clarified that *Rosmarinus officinalis L.*essential oil showed different effects for gene expression for the three selected isolates(8,24and 40).

Introduction

The bacteria in the genus *Klebsiella* are Gram-negative, facultative, anaerobic, non-motile rods with a conspicuous polysaccharide capsule (Holt *et al*., 2015). *K. pneumoniae* expresses type 1 and type 3 fimbriae of two fimbrial adhesions (Stahlhut *et al.,*2010). All members of Enterobacteriacae possess type 1 fimbriae, which are necessary for *K. pneumoniae* to produce urinary tract infections (UTI). They use the adhesion, which is found on the tip of the fimbriae and identifies mannose-containing glycoproteins found on many mammalian host tissues, to exert their adhesion characteristics, and their expression is phase variation encoded by operon fim (Schembri *et al.,*2005). It has been discovered that type 3 fimbriae are essential for the formation of *K. pneumoniae* biofilms and their attachment to medical equipment. Extracellular matrix, such as that seen on exposed tissues and coating indwelling devices, has been identified to selectively bind to *MrkD.Rosmarinus officinalis L.,* a Mediterranean shrub member of the Lamiaceae family, one of the biggest and most illustrious families of flowering plants. Rosemary has been widely used in traditional medicine as well as in cooking, notably to alter and enhance flavors. It is also a highly regarded medicinal plant for colds, rheumatism, and pain in the muscles and joints (Zhang, 2014). Carnosic acid, along with essential oil, is the bioactive ingredient that has been studied the most with roughly 30% of the studies total. Carnosol, rosmarinic acid, and ursolic acid came in second with 35% of the studies total (Andrade *et al.,* 2018).

Materials and Methods

Isolation and identification of Bacterial isolates

Eleven *K.pneumoniae* isolates was recovered from different clinical samples and identified using vitek 2 system then confirmed in a molecular method using *16SrRNA* gene specific primer as mentioned by (Jabar *et al*.,2022).

Determination of Minimal Inhibitory Concentrations of rosemary essential oil

The modified microbroth dilution method was used inorder to investigate MIC of rosemary against the tested isolates (strong biofilm producers and MDR)(Jabar *et al*.,2022) , ready-made oil solutions of 100 % (*Rosmarinus officinalis L.)* were employed as the stock solution. The oil concentrations were (**5×10^5, 2.5×10^5, 1.25×10^5, 6.25×10^4, 3.12×10^4, 15×10^3, 7×10^3, 3×10^3) µg/ml** prepared using DMSO**. .** Microtiter plate was used ,the first well(contain brain heart broth) received 100 µl of operating stock. One hundred µLwere transferred from the first well to the second and carefully mixed**.** From the eighth well, 100 µL were collected and then wasted. To each of the wells, 100 µL of bacterial suspension was introduced (to 8).The plate was incubated at 37°C for 18 to 24 hours.After another four hours at 37 °C, 3µL of resazurin (0.15 mg/ml) was added to each well. Color variations were observed and noted. The lowest concentration prior to color change was identified as MIC. The Clinical and Laboratory Standards Institute determined MIC readings (CLSI, 2017).

Antibiofilm activity of sub MIC rosemary essential oil against *K.pneumoniae* isolates

Strong biofilm-producing and MDR isolates(11 isolates) were grown in brain heart infusion broth for 24 hours at 37°C.Microtiter plate method was used to study the antibiofilm activity of rosemary oil,As mentioned in biofilm formation experiment by Jabar *et al*. (2022) this step was added, each well was received a volume (100 µl) of sub-MIC rosemary essential oil,incubated for 24 hours at 37°C under aerobic conditions.

All plates were moderately washed three times with distilled water after incubation, the plates were dyed with 200 µl 1% crystal violet solution. A 200 µl of free ethanol was used to resolubilize the adhering cells for 10 minutes. The microplate ELISA reader at 630 nm measured the optical density of each well. The percentage of inhibition in biofilm formation was measured using the following formula:

Biofilm inhibition (%) = (Control OD- Test OD / Control OD) ×100

Real Time-qPCR

RNA was extracted and purified from *K. pneumoniae* by using an RNA purification kit (GoTaq® 1-Step RT-qPCR System, MgCL2, Nuclease Free Aquatic, Quantifluor RNA System) according to the Manufacturer Company and then RNA concentration was measured by using (Quantus Florometer). Three isolates out of 11 isolates their numbers are (8,24 and 40) all of which isolated from urine samples ,were grown in brain heart infusion broth as a control in addition to isolates were treated with rosemary oil (sub MIC **15×10^3 , 3.12×10^4** µg/ml) since we chose two isolates

their numbers (24 and 40) that MDR and strong biofilm producers and one isolate that not MDR but strong biofilm producer their number(8). *Fimh* ,*mrkd* genes were tested and 16SrRNA gene used as house keeping or reference gene.

The steps of qRT-PCR were used as follows:

1. Combine the GoTaq® 1-Step RT-qPCR system, template RNA, primers, and DNase/Rnase-free water before quickly centrifuging to extract the bottom-of-the-PCR-tube solutions as shown in table (1).

:The components of the Reaction mixture in qRT-PCR Table(1)

No.	Master mixComponent	Volume(1 sample)
1	qPCR Master Mix	5µl
2	RT mix	0.25µl
3	MgCl2	0.25µl
4	Forward primer	0.5 µl
5	Reverse primer	0.5 µl
6	RNA	1 µl
7	Nuclease free dH2O	2.5 µl
	Final volume	**10µl**

2. In PCR tubes, RNA samples were added to the reaction setup, flat caps or optically clear film was used to shut the tubes, and then a gentle vortex was employed to make sure the reaction components were thoroughly mixed. The tubes were dried to get rid of any air bubbles, and the mixture for the reaction was assembled at the bottom of the container (table 2).

3. The thermal cycler was adjusted so that qPCR amplification actually occurs after cDNA synthesis.

Table(2) :Real-Time PCR Program

Steps	°C	m: s	Cycle
RT. Enzyme Activation	**37**	**15:00**	**1**
Initial Denaturation	**95**	**05:00**	
Denaturation	**95**	**00:20**	**40**
Annealing	**57,58, or 62**	**00:20**	
Extension	**72**	**00:20**	

Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method

With the aid of relative quantification, the expression levels were determined. Cycle thresholds (CT), fold changes, and differences between treatment groups and each gene's calibrators were all calculated (Livak and Schmittgen, 2001). Results were standardized using the expression of the *16S rRNA* gene as shown below:

Fold gene expression $=2^{\wedge -\Delta\Delta CT}$

ΔCT =CT target gene - CT House Keeping gene

$\Delta\Delta CT$ =ΔCT Treated gene - ΔCT untreated gene

Results and Discussion

Eleven *K. pneumoniae* isolates that are MDR and strong biofilm producers were taken as mentioned in current study done by Jabar,*et al.*(2022).The MIC of rosemary essential oil against *K. pneumoniae* was determined. The results showed that the MIC for the 11 isolates were **6.25×10^4**for 3 isolates (27%) and **3.12×10^4**for 6 isolates (54%) and **15×10^3** for 2 isolates (18%) as shown in table (3).

Table(3): MICs of *Rosmarinus officinalis* L.Essential Oil against *K. pneumoniae*

Isolates	MIC µg/ml
KP 3	**3.12×10^4**
KP 6	**6.25×10^4**
KP 12	**3.12×10^4**
KP 21	**3.12×10^4**
KP 22	**15×10^3**
KP 24	**3.12×10^4**
KP 25	**3.12×10^4**
KP 29	**15×10^3**
KP 30	**3.12×10^4**
KP 31	**6.25×10^4**
KP 40	**6.25×10^4**

The MIC value of EO was 10^4µg/ml on 24 (83 %) of the isolates, 0.5×10^4 µg/ml on 4 (14 %), and 2×10^4µg/ml on 1 (3 %), according to local research conducted by Abdulhasan, (2015). When using Rosemary ethyl acetate extract against *K. pneumoniae* similar effects were found. The examined bacteria had varying intrinsic degrees of resistance to antimicrobials, which might account for the reported differences in MIC values among isolates, whereas aqueous and crude extracts inhibited the bacteria at greater concentrations (Abdel-Massih *et al.,* 2010).

Sample	Mean of absorbance before treatment	Mean of absorbance after treatment	Biofilm Inhibition ratio %
KP3	0.279	0.207	25.8
KP6	0.397	0.359	9.6

KP12	0.459	0.115	74.9
KP21	0.397	0.213	46.3
KP22	0.418	0.115	72.5
KP24	0.603	0.218	63.8
KP25	0.432	0.363	16.0
KP29	0.516	0.226	56.2
KP30	0.354	0.299	15.5
KP31	0.459	0.345	24.8
KP40	0.608	0.195	67.9
p-value	**0.000**		

Eleven *K. pneumoniae* isolates were also selected to detect the effect of 1/2 MIC or sub MIC concentrations of Rosemary oil on biofilm formation in microtiter plate method. Results show that there were a significant differences ($P \leq 0.05$) in OD values of biofilm formation between *K. pneumoniae* isolates before and after treatment with Rosemary oil, and the biofilm inhibition ratio % was also determined as demonstrated in tables (4) & figures(1)

Table(4):Antibiofilm activity of *Rosmarinus officinalis* L. essential oil against selected isolates of *K. pneumoniae*

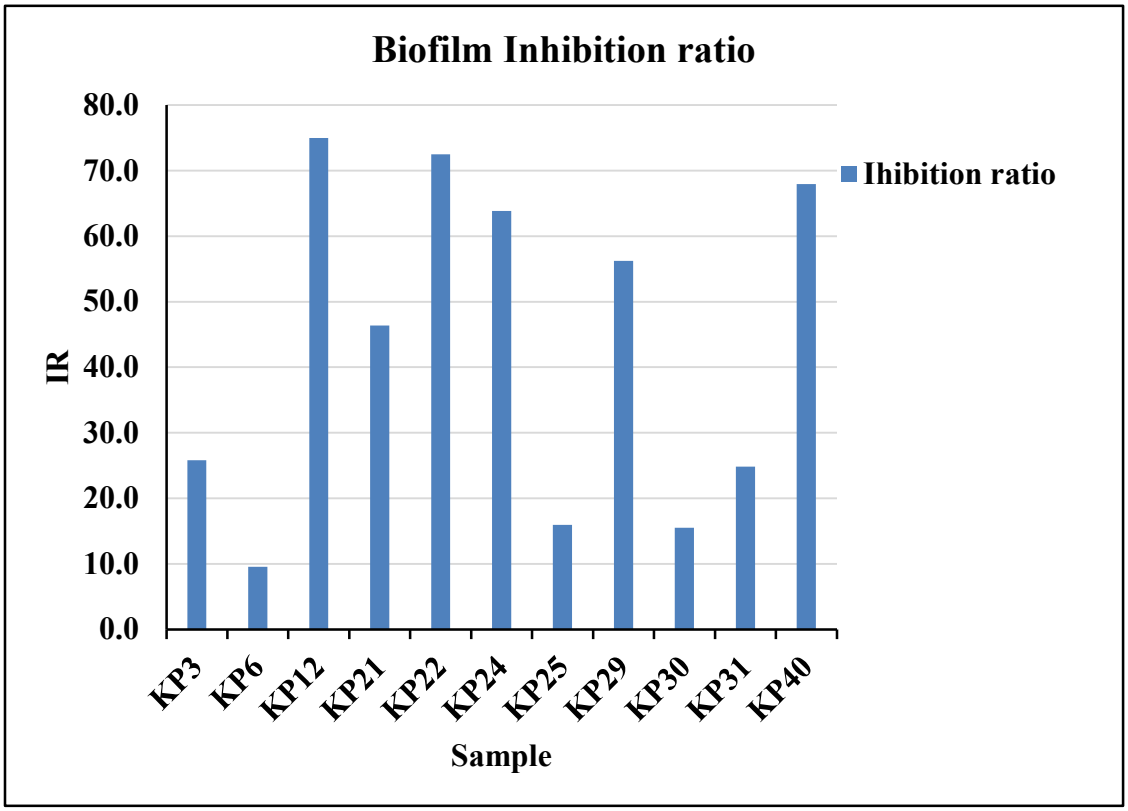

Figure (1): Biofilm Inhibition ratio of rosemary essential oil against selected isolates of *K. pneumoniae*

Our finding is consistent with the results of local study done by Abdulhasan, (2015) who recorded that the effect of MIC and 1/2 MIC of antibiotics and rosemary essential oil used alone and in combinations found significant differences ($P \leq 0.05$) in biofilm reduction percentage of all isolates of *K.pneumoniae* in comparison to control. In strong biofilm formation, the highest percentage of reduction was observed when used a combination of 1/2 MIC of gentamicin and 1/2 MIC rosemary essential oil (50%) and 1/2 MIC rosemary essential oil (51%) alone that biofilm formation was reduced from 0.36 to 0.18 and 0.36 to 0.175. Sahal *et al.* (2016) obtained that 170 µl/mL of lemon essential oil caused 48.3% (±13.7) decreases in biofilm formation amounts of strongest biofilm forming *K. pneumoniae.*

Using a one-step RT-PCR approach, quantitative real-time PCR was carried out to ascertain the impact of sub-MIC rosemary essential oil on the expression of *mrkd* in *K. pneumoniae* isolates. The results demonstrated no change in the *mrkd* gene under the rosemary oil effect for the first isolate(24) and an increase in the expression level (up regulation) of the *mrkd* gene in the expression level for the second isolate(40) using the livak equation 2^ΔΔct which is a simple and direct method for measuring relative changes in gene expression in real-time quantitative PCR experiments (Livak and Schmittgen, 2001).While the expression of *mrkd* gene for the third isolate (8) was decreased to 0.707 (down regulation) for the treated sample in comparison to the control.

The result of qRT- PCR in this study revealed that the expression of *mrkd* gene in the existence of oil for the first isolate (24) at a concentration of sub MIC (**15×10^3** µg/ml) was 1.2 , while the expression of the gene for the second isolate(40) at a concentration of sub MIC (**3.12×10^4**µg/ml) was 9.189 compared to control samples as shown in table (5) .

Table (5) : Ct values and fold change of *mrkd* gene expression of *K. pneumoniae* selected isolates that were treated with rosemary essential oil

Sample	16SRNA	mrkD	ΔCT	ΔΔCT	Folding
24 Control	17.1	19.1	2.0	0.0	1.0
24oil treatment	15.4	17.1	1.7	-0.3	1.2
40 Control	17.0	24.3	7.3	0	1.0
40oil treatment	16.3	20.3	4	-3.3	9.189
8 control	16.2	20.2	4	0.0	1.0
8 oil treatment	17	21.5	4.5	0.5	0.707

The result of qRT- PCR in this study also revealed that the expression of *fimH* gene in the existence of oil for the first isolate (24) at a concentration of sub MIC (**15×10^3** µg/ml) was decreased to 0.870 in comparison to control(not treated with oil), while the expression of the gene for the second

isolate(40) at a concentration of sub MIC (**3.12×10^4**µg/ml) was not affected ,while the third isolate (8)showed a decrease in the expression of *fimh* gene to 0.812 (down regulation) compared to control samples as shown in table (6).

Table (6) : Ct values and fold change of *fimh* gene expression of *K. pneumoniae* selected isolates that were treated with rosemary essential oil

Sample	16SRNA	fimH	ΔCT	ΔΔCT	Folding
24 Control	17.1	18.9	1.8	0.0	1.0
24oil treatment	15.4	17.4	2	0.2	0.870
40 Control	17	19.5	2.5	0.0	1.0
40oil treatment	16.3	18.2	1.9	-0.6	1.515
8 control	16.2	18.2	2	0.0	1.0
8 oil treatment	17	19.3	2.3	0.3	0.812

From this current data, the biofilm formation in microtiter plate decreased when we used sub or 1/2 MIC rosemary essential oil and when we study the gene expression for type 1 & type 3 fimbriae we can conclude that the two isolates(24 &40) appear different attitude even it had the same isolation source (urine) this may be due to that each isolate of which was MDR and strong biofilm producers but the other isolate (8) not MDR but strong biofilm producer, this may be needed further investigation for other fimbrial genes on a molecular level. Our work may be done for the first time, and we will not obtain global or local results in order to compare it with our data, at least for *fimh* gene expression. However, several genes are coding for fimbriae, each determining specific functions in virulence and biofilm formation.

Conclusion

Rosemary essential oil inhibit the biofilm formation for all the tested *k.pneumoniae* isolates regardless of their origin or source of isolation.*R. officinalis L*.essential oil showed different effects for gene expression for the three selected isolates: isolate no.24 was not affected for the *mrkd* adhesin gene but showed a decrease in *fimh* adhesin gene expression, while isolate no. 40, the expression was increased for the first gene(*mrkd*)and not affected for the second gene(*fimh*).Isolate no.(8) showed a hopeful data since the expression of two tested genes was down regulated in the treated sample in comparison to the control.

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