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The Biological Effect of *Rosmarinus officinels* L. Essential Oil on Biofilm Formation and Some Fimbrial Genes (*fimH-1* and *mrkD*) of *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae possesses many virulence factors and survival strategies to persist and overcome host defenses; one of these strategies is biofilm formation. Therefore, the aims of this study was to determine the antibacterial and antibiofilm effect of *Rosmarinus officinels* L. essential oil (EO) and its effect on the genes encoding of fimbrial adhesions. The antimicrobial activity was investigated by MIC. The ability to form biofilm as well as inhibition of initial cell attachment and biofilm formation was performed. PCR was carried out to detect *fimH-1* and *mrkD* genes of type 1 and type 3 fimbrial adhesions at different time of incubation. The study revealed that MIC value of EO was 10^4 $\mu\text{g/ml}$ on 24 (83%) of isolates, 93% of them produced biofilm. Fifty percent reduction in biofilm formation was observed in 10% of isolates at concentration 10^4 $\mu\text{g/ml}$ and increased to 45% when used 1.5×10^4 $\mu\text{g/ml}$ of EO. PCR product of *fimH-1* was detected at 24 h but absence at 0 and 4 h while *mrkD* product found in all incubation time. In conclusion, Rosemary EO had antibacterial and antibiofilm activity against *Klebsiella pneumoniae*. Moreover, it affected the type 1 fimbriae at gene level probably by mutation during initial attachment of biofilm formation.

Keywords: *Klebsiella pneumoniae*, Rosemary, biofilm, type 1 and 3 fimbriae.

التأثير الحيوي للزيوت الطيارة لنبات اكليل الجبل *Rosmarinus officinels* L. على تكون الغشاء الحيوي وبعض جينات الخمل (*fimH-1* and *mrkD*) لبكتريا *Klebsiella pneumoniae*

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الخلاصة

Klebsiella pneumoniae بكتريا تمتلك عوامل فوعة متعددة واستراتيجيات للبقاء على الاستمرار والتغلب على دفاعات المضيف؛ واحدة من هذه الاستراتيجيات هو تشكيل الغشاء الحيوي. ولذلك هدفت هذه الدراسة لتحديد التأثير المضاد للبكتريا والمضاد للغشاء الحيوي للزيوت الطيارة لنبات اكليل الجبل وتأثيره على جينات التصاقات الخمل. استعملت طريقة اقل تركيز مثبط من الزيت للتحري عن النشاط المضاد للبكتريا. واختبرت القدرة على تشكيل الغشاء الحيوي وكذلك تثبيط الارتباط الاولي وتثبيط تشكيل الغشاء الحيوي. استخدمت تقنية تفاعل سلسلة البلمرة للكشف عن جينات *fimH-1* و *mrkD* المسؤولة عن التصاقات الخمل من نوع 1 ونوع 3 في أوقات مختلفة من الحضانة. وكشفت الدراسة أن 24 (83%) من العزلات لها اقل تركيز

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مثبط من الزيت يساوي 10^4 ميكروغرام / مل و 93% منها منتجة للغشاء الحيوي. وقد لوحظ انخفاض خمسين في المئة في تشكيل الغشاء الحيوي في 10% من العزلات بتركيز 10^4 ميكروغرام / مل وارتفعت إلى 45% عند استخدامها $1,5 \times 10^4$ ميكروغرام / مل من الزيوت الطيارة. تم الكشف عن جين *fimH-1* عند 24 ساعة مع غيابه عند 0 و 4 ساعات بينما جين *mrkD* موجودة في كل فترات الحضانة. في الختام، الزيوت الطيارة نبات اكليل الجبل لديها فعالية مضادة للبكتريا لتكوين الغشاء الحيوي من بكتريا *Klebsiella pneumoniae*. وعلاوة على ذلك، فإنه يؤثر على التصاقات الخمل من نوع 1 على مستوى الجينات ربما عن طريق حدوث طفرة في الجين خلال الارتباط الاولي لتشكيل الغشاء الحيوي.

Introduction

Klebsiella pneumoniae is an important pathogen in both community and hospital setting and it is second only to *Escherichia coli* in nosocomial Gram negative bacteremia, urinary tract infection effecting catheterized patient (70%), sepsis and pneumonia [1]. One common survival strategy employed by *K. pneumoniae* is to form a biofilm [2].

Biofilm is a structured community of bacterial cells that involves two stages of biological process controlled by surface adhesions to biotic or abiotic surface and cell to cell communication via quorum sensing to accumulate in multilayered clusters protected and/or coated matrix [3].

Fimbrial adhesions facilitate adherence to specific tissue surfaces (biotic) as well as abiotic surfaces. *K. pneumoniae* express two fimbrial adhesions type 1 and 3 fimbriae [4]. Type 1 fimbriae are essential for the ability of *K. pneumoniae* to cause urinary tract infection (UTI) and found on all members of Enterobacteriaceae. They exert their adhesive properties by virtue of the Fim H adhesion located on the tip of fimbriae that recognizes mannose-containing glycoproteins present on many mammalian host tissues and their expression is phase variation encoded by operon *fim* [1].

Type 3 fimbriae are encoded by *mrk* operon, which their genes cluster may reside on multiple genomic locations including chromosome, conjugative plasmids and within a composite transposon which support spread of these genes between gram negative bacteria by lateral gene transfer [5]. It has been shown to promote biofilm formation on biotic as well as on abiotic surfaces while type 1 are essential for the ability of bacteria to cause UTI [4]. Type 3 fimbriae mediate binding to extracellular matrix proteins such as collagen [6] and epithelial cells of respiratory and urinary tracts [7].

As a result of biofilm formation, the bacteria persistent infections with chronic inflammation and develop resistance against antibiotics especially in extended spectrum beta lactamases (ESBL) strains. Infections caused by biofilm forming bacteria are often difficult to treat and have resistance to antibiotics up to 1000 fold decrease in susceptibility in comparison with planktonic bacteria [8]. The increasing antibiotic resistance lead to increase attention to discover an alternative therapeutic molecules that can inhibit biofilm formation and bacterial growth [3].

Rosemary (*Rosmarinus officinalis* L.) has been widely used in traditional medicine, cosmetics and flavoring agent in foods. Rosemary essential oil has antibacterial effect for both Gram negative and positive bacteria, it's also antifungal, antioxidant, antimutagenic and shows cytotoxic activity [9]. The major compounds of essential oil (EO) determine their biological effect that can cause the damage of wall and membranes, inhibition of protein synthesis, interference with metabolism or interference with DNA/RNA synthesis and function [10].

Therefore, the aims of this study were to determine the antibacterial and antibiofilm effect of Rosemary essential oil and its effect on the genes encoding of fimbrial adhesions respectively.

Materials and methods

Twenty nine isolates of *Klebsiella pneumoniae* were isolated from different specimens (urine, blood, wound and sputum) on MacConkey agar (Himedia/ India) at 37°C. All isolates were confirmed using API-20E test kit (Bio-Merieux, France) according to the manufacture instructions in central health laboratory. These isolates were previously identified using molecular techniques as extended spectrum beta lactamases (in press).

Preparation of Essential oil of *R. officinalis*

The EO of Rosemary was isolated from air dried leaves (250g) by Cleavenger hydrodistillation method. The plant material with D.W (1.2L) was boiled for 3h, the EO was kept at 4°C until use [11, 12]

Determination of minimal inhibitory concentration (MIC)

The MIC values of Rosemary EO were evaluated using microdilution method in 96 well microtitre plate as recommended by the clinical and laboratory standard institute [13]. EO of Rosemary was dissolved in DMSO (2×10^4 µg/ml) then two fold serial dilutions were prepared with brain heart infusion broth (Himedia/ India) in a final concentration ranging from 2×10^4 -156 µg/ml and the bacterial inoculums were added at final concentration 1×10^6 cell/ml, bacteria and media control were prepared. The results were recorded after incubation at 37°C for 18 h and measuring at 630nm with an ELISA reader (BioTek). MIC (lowest concentration of the EO that inhibits the growth of bacteria) was determined by taking absorbance of the tested wells that did not exceed the value of the absorbance media control [14].

Biofilm formation assay

Biofilm was performed in 96 well microtitre plate as described by Wojnicz *et al.* [15] with some modification. Briefly, *K. pneumoniae* isolates were subcultured in brain heart infusion broth (Himedia/ India) over night at 37°C. 200 µl of bacterial cultures (O.D₅₄₀ 0.64 = 10^8 cell/ml) were transferred to each well in triplicate then incubated 24h at 37°C and negative control contained media only. The media was removed and washed three times with phosphate puffer saline then 25µl of crystal violet (1% w/v) was added to the wells for 15 min at room temperature. Crystal violet was then removed and washed three times with PBS. The crystal violet inside the cells was dissolved by absolute ethanol (100 µl) and the absorbance was measured by an ELISA reader. After comparing the optical density (O.D) of biofilm to the control and according to the readings, the isolates were classified as follows: O.D ≤ O.D_c no biofilm producer, O.D_c < O.D ≤ 2 × O.D_c weak biofilm, 2 × O.D_c < O.D ≤ 4 × O.D_c moderate and 4 × O.D_c < O.D strong biofilm.

Inhibition of initial cell attachment assay

The first step in biofilm formation is attachment of cell to the surfaces. The assay was performed in 96 well microtitre plate [3], 50µl of bacterial inoculums was added at final concentration 1×10^6 cell/ml and incubated for 4 h at 37°C, then 50µl of two fold serial dilutions of EO in brain heart infusion broth (Himedia/ India) at concentrations ranging from 2×10^4 -156 µg/ml were added to wells. Bacterial and medium control were prepared and incubated at 37°C for 18 h. The crystal violet assay was performed as mentioned above. The concentration of EO that inhibited the initial cell attachment to microtiter plate was determined according to the absorbance of media control.

Biofilm inhibition assay

The antibiofilm activity of Rosemary EO was tested on *K. pneumoniae* biofilm using 96 well microtitre plate as described by Selim *et al.* [11] with modifications. Biofilm formation was achieved by adding 100 µl of bacterial suspension (10^8 cell/ml) then incubated at 37 °C for 24 h. The EO of Rosemary was added at a final concentration of 10^4 and 1.5×10^4 µg/ml in the wells. The medium without extract was used as control and the plate was incubated further at 37 °C for 24 h. Following incubation with the extract, the crystal violet assay was performed as discussed above. The percentage of reduction in biofilm formation was measured using the formula [16]:

$$\% \text{ Reduction} = \frac{\text{Control OD} - \text{Test Sample OD}}{\text{Control OD}} \times 100$$

DNA Extraction

Boiling method was used for extraction of genomic DNA from *K. pneumoniae* isolates as the method described by Ahmed *et al.* [17], with modifications as follow: bacterial cells were harvested in one ml of Tris-EDTA (TE) buffer and centrifuged at 12000 g for 2min. Pellet was re-suspended in 100 µl of sterile distilled water and boiling at 100°C for 10 min, cooled on ice then centrifuged at 10000 g for 10 min. The supernatant was stored at - 20 °C until use. *K. pneumoniae* isolates that showed percentage of reduction in biofilm formation above 50% were chose for treating with 1.5×10^4 µg/ml of Rosemary EO for different incubation times. For each isolate, Rosemary EO was added to bacterial inoculums at the beginning of incubation (0 time), another two tubes contained bacterial inoculums were treated with Rosemary EO after 4 h and 24h of incubation. The genomic DNA of Rosemary EO treated isolates at different incubation time were extracted as mentioned above.

PCR amplification

FimH-1 and *mrkD* genes were detected by PCR using specific primers Table-1. The PCR mixture set up in 20 μ l total volume consisting of 5 μ l of premix Accupouer (Bionear, Korea), 10 pico/ μ l of each primer and 100 ng/ μ l of DNA template. The thermal programme was optimized and performed in master cycler (Eppendorf, Germany) as follows: 4min at 94 °C, then 35 cycles of 30sec at 94 °C, 30 sec at 52.9 °C for *fimH-1* primers and 46.8 °C for *mrkD* primers as well as 50 sec at 72 °C then final elongation step at 72 °C for 7 min.

Statistical analysis

All data were analyzed using the SPSS IBM version 20. Least significant differences (LSD) was used for comparison of essential oil concentrations on type of biofilm formation. F-test was used to analyse the role of essential oil concentration on biofilm formation. The $P \leq 0.05$ was considered statistically significant.

Table 1-The primers and their sequences used in PCR for detection of *fimH-1* and *mrkD* genes in *K. pneumoniae*.

Primer	Gene	Sequence 5' - 3'	Product size (bp)	references
fimH-F	<i>fimH</i>	ATGAACGCCTGGTCCTTTGC	688	[4]
fimH-R		GCTGAACGCCTATCCCCTGC		
mrkD-F	<i>mrkD</i>	CCACCAACTATTCCCTCGAA	240	[16]
mrkD-R		ATGGAACCCACATCGACATT		

Results and Discussion

K. pneumoniae causes sever nosocomial infections because it's difficult to be treated and lead to persistent of infection, despite the appropriate treatment by antibiotics. Their antibiotic resistance enzymes (ESBLs) resulting from wide spread use of antibiotics that threatens the global public health. Therefore, plant extracts used as an alternative therapy for treatment of pathogenic bacteria because they possessed antivirulence factor properties and can easily inhibit biofilm formation.

The MIC value of Rosemary EO against *K. pneumoniae* was determined by broth microdilution method. It was revealed that MIC value of EO was 10^4 μ g/ml on 24 (83%) and 0.5×10^4 μ g/ml on 4 (14%) as well as 2×10^4 μ g/ml on 1 (3%) of the isolates. Similar results were obtained when used ethyl acetate extract of Rosemary against *K. pneumoniae*. Reported variations in MIC value among isolates might return to the presence of different intrinsic levels of tolerance to antimicrobials with tested bacteria, while aqueous and crude extracts inhibited the bacteria at higher concentration [18]. *Quercus brantii* ethanol extract did not inhibit *K. pneumoniae* due to the presence of capsule, whereas Cinnamon and Green tea exhibited the highest antibacterial activity against this bacterium [19].

The advantages of using Rosemary EO instead of antibiotics include: 1. Most EO derived from plant consist of chemical compounds that easily diffuses a cross cell membrane to induce biological reactions [20]. 2. EO have complexes and comprise compounds in variable properties that help not to create resistance [14].

K. pneumoniae have the ability to produce biofilm and according to the results, 93% of isolates produced biofilm and were classified as 8, 19 and 2 isolates strong, moderate and weak biofilm producers respectively. Also, the biofilm formation in strong producer isolates was 3.1 fold greater than weak and 1.6 fold than moderate isolates. The ability of biofilm formation in this study was in agreement with published studies [21, 22]. The high rate of biofilm formation might be related to phenotypes and genes involved in biofilm such as capsule, lipopolysaccharide and fimbriae [5].

The first step in the formation of a biofilm is the attachment of planktonic cells onto biotic or abiotic surface, therefore the inhibition of initial cell attachment might be the starting point in combating the formation of these resistant biofilms. Thus, in further experiment, Rosemary EO showed antimicrobial like activity against initial attachment of *K. pneumoniae* biofilm when 10^4 μ g/ml of EO (equal to MIC) was used. Selim *et al.* [11] reported the inhibition of cell attachment to a substrate was easier to achieve than inhibiting the growth of established biofilm.

The results showed a considerable reduction in biofilm formation when treated with different concentrations of Rosemary EO as shown in figure-1. Fifty percent reduction in biofilm formation was

observed in 10% of isolates at concentration 10^4 $\mu\text{g/ml}$, and this percentage of isolates were increased to 45% when 1.5×10^4 $\mu\text{g/ml}$ of EO was used.

Although both concentrations showed significant differences ($P \leq 0.05$) in biofilm formation reduction, but 1.5×10^4 $\mu\text{g/ml}$ had high effect on strong and moderate biofilm formation ($P \leq 0.001$) in comparison to weak producer. This might be related to the surface chemistry of these producers as mentioned in Naveed *et al.* [20] study on *Salmonella typhi*, they noted that EO have double activity against biofilm producing bacteria than non-producer one.

Different EO showed different reduction percentages in *K. pneumoniae* biofilm formation included Eucalyptus oil (82%), Tulsi oil (48.7%), Garlic oil (47.3%) and Neem oil (34.3%) [16].

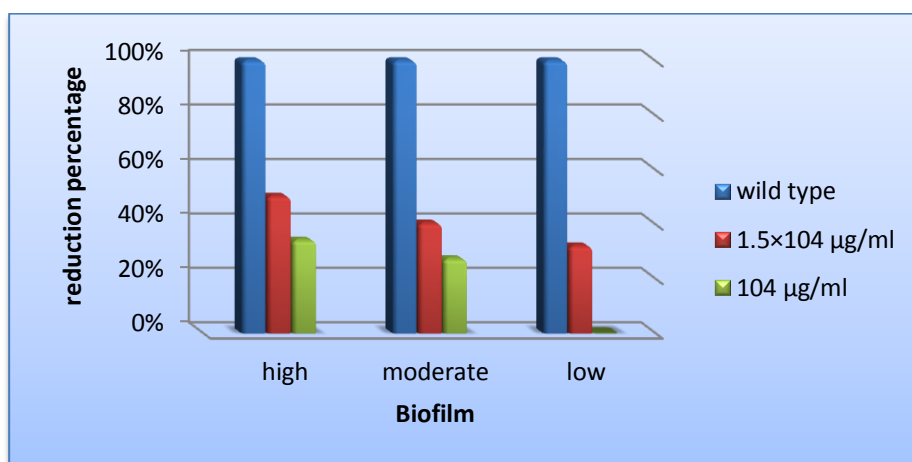


Figure 1-Reduction percentage of *K. pneumoniae* biofilm formation by Rosemary EO.

The differences in biofilm reduction percentage might belong to the differences of EO incubation time, 24h may be not enough and need more time to obtain high reduction rate as recorded by Bilcu *et al.* [10]. Also, the reduction percentage was lowered due to multilayer defense mechanism [23] and to the presence of physical barriers that is related to mucoidy, which lead to biofilm resistance [8]. In this study, the concentrations used to inhibit 50% of biofilm formation were one fold or less than MIC value which was preferable than using high concentration of antibiotics. Biofilm resistant to antibiotics in about 100-1000 fold above MIC of planktonic cells because all pathogen harbor a mechanism of multidrug resistance which expresses it only when growing as biofilm [22]. The reason for inhibiting biofilm at low concentration of natural compounds due to the inhibition of receptors and molecules involved in quorum sensing pathway which is required for biofilm formation [3].

Polymerase chain reaction technique was carried out to detect *fimH-1* and *mrkD* genes responsible for expression of type 1 and type 3 fimbrial adhesions, the results showed that all isolates (100%) possessed these genes which is in agreement with other published data [21, 24, 25].

Three isolates of *K. pneumoniae* exhibited above 50% reduction in biofilm formation were chosen to investigate the effect of Rosemary EO on type 1 and type 3 fimbriae in biofilm formation that added at different time of incubation (0,4 and 24h).

After incubation, the extracted DNA was amplified and the PCR amplified product (688bp) of *fimH-1* was found at 24 h but was absent at 0 and 4 h of incubation time Figure-2. However, these results may give point for mutation of *fimH* leading to the absence of this virulence gene [26]. *fimH* is encoded for Fim H adhesion that located on the tip of fimbriae, therefore exposure of *K. pneumoniae* isolates to Rosemary EO at the beginning of incubation (0 and 4h) might affect Fim H adhesion during initial attachment in biofilm formation.

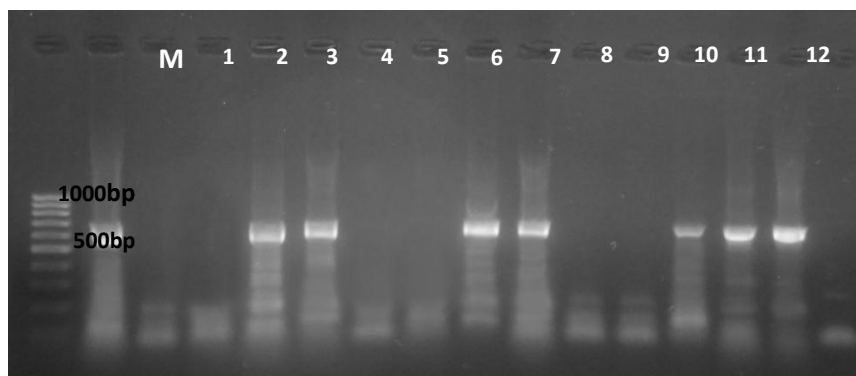


Figure 2-PCR amplification of *fimH-1* (688bp) in *K. pneumoniae* isolates, Agarose (1.5%), 5 V/cm for 45 min and visualized under U.V after staining with ethidium bromide. Lanes 1-4: **Isolate 1** (control, 0, 4h, 24h). Lanes 5-8: **Isolate 2** (control, 0, 4h, 24h). Lanes 9-12: **Isolate 3** (control, 0, 4h, 24h). Lane 13-14 other *K. pneumoniae* isolates. C: negative control. M: 100bp DNA marker. **Isolate 1, 2, 3**: produce 57% , 54% and 52% reduction in biofilm formation respectively.

mrkD amplified product (240bp) was present in all incubation time of three studied isolates Figure-3, that means Rosemary EO did not effected the mentioned gene. Type 3 fimbriae are important in biofilm formation in comparison to type 1 fimbriae. Other studies showed that type 3 fimbriae contribute to biofilm formation in biotic and abiotic surfaces and important for cell to cell adherence [7, 27].

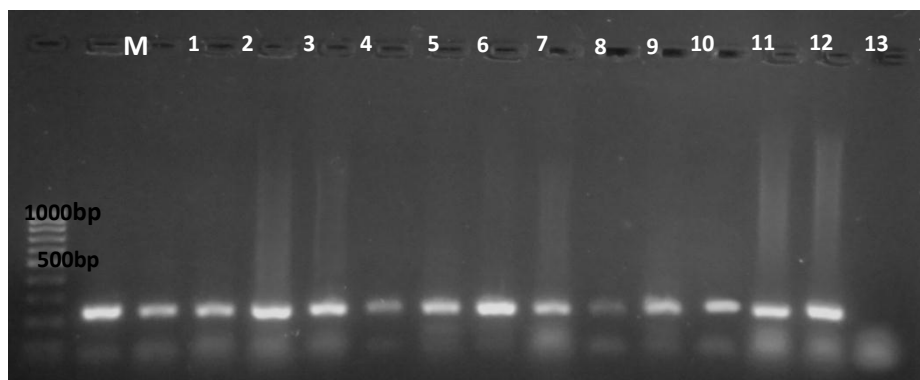


Figure 3 - PCR amplification of *mrkD* (240bp) in *K. pneumoniae* isolates, Agarose (1.5%), 5 V/cm for 45 min and visualized under U.V after staining with ethidium bromide. Lanes 1-4: **Isolate 1** (control, 0, 4h, 24h). Lanes 5-8: **Isolate 2** (control, 0, 4h, 24h). Lanes 9-12: **Isolate 3** (control, 0, 4h, 24h). Lane 13-14 other *K. pneumoniae* isolates. C: negative control. M: 100bp DNA marker. **Isolate 1, 2, 3**: produce 57% , 54% and 52% reduction in biofilm formation respectively.

In conclusion, the findings of the current study indicated that Rosemary EO had antibacterial activity and considerable antibiofilm activity against *Klebsiella pneumoniae*. Moreover, it affected the type 1 fimbriae at gene level probably by mutation during initial attachment of biofilm formation.

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