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Research article

Effect of sub-inhibitory and inhibitory concentrations of some antibiotics and rosemary essential oil (*Rosmarinus officinalis L.*) on biofilm formation of *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae have an ability to form biofilm as one of strategies to persist and overcome host defenses. The study aims to evaluate the effectiveness of rosemary essential oil alone and in combination with some antibiotics against biofilm of *K. pneumoniae* isolated from urine. The antibiotics resistance pattern by disc diffusion method and minimal inhibitory concentration (MIC) of gentamicin, ciprofloxacin, amoxicillin, trimethoprim/ sulfamethoxazole, cefotoxime and rosemary essential oil were determined. The ability to form biofilm as well as inhibition of biofilm formation of *K. pneumoniae* was performed. MICs 128, 0.25, 768, 64, 384 and 10^4 µg/ml were used. The effect of MIC and 1/2 MIC of antibiotics and rosemary essential oil that used alone and in combinations found significant differences ($P \leq 0.05$) in biofilm reduction percentage of all isolates in comparison to control. In conclusion, the combination of antibiotics with rosemary essential oil effect negatively on biofilm of *K. pneumoniae*.

Keywords: Biofilm, *Klebsiella pneumoniae*, Rosemary essential oil

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INTRODUCTION

Klebsiella pneumoniae is one of the most important pathogens causing opportunistic infections, such as pneumonia, sepsis, and inflammation of the urinary tract [1]. The important virulence factors and strategies that playing a role in the severity of *K. pneumoniae* infections are capsular polysaccharides, type 1 and type 3 pili, factors involved in aggregative adhesions, siderophores [2] and biofilm, more than 60 % of infections and up to 80 % of chronic infections have bacteria grown in biofilms [3].

Several factors required for biofilm formation have been identified in *K. pneumoniae* clinical isolates from the gastrointestinal tract, pneumonia and urinary tract infections (UTIs) [4]. Type 1 and type 3 pili have a role in mediating *K. pneumoniae* colonization which able bacteria to attach the uroepithelial surface and cause urinary tract infections [5]. Microorganisms growing in a biofilm are highly resistant to antimicrobial agents by one or more mechanisms. The development of biofilms by pathogenic bacteria is believed

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to play an important role in evade of host defense mechanisms, communication between bacterial cells and protection against antibiotic action [6].Various antibiotics have been used to treat infections caused by *K. pneumoniae* but appearance of resistant strains to commercial antibiotics lead to difficult treatment of infections [7]. Aminoglycosides and fluoroquinolones have been widely used as antibacterial against members of enterobacteriaceae family. Antibiotics might kill the planktonic bacteria in vivo but fail to eliminate bacterial cells aggregate in a biofilm [8]. Also, the subinhibitory of minimum inhibitory concentrations (sub-MICs) of certain antibiotics can alter the morphology and virulence of bacterial species [9].

Rosemarinus officinalis essential oil is an important for medicinal treatment and its powerful as antibacterial, cytotoxic, antimutagenic and antioxidant properties. Characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable [10].

Combination therapy may play a crucial role in eradicating resistant *K. pneumoniae* that can broaden the spectrum of antibacterial activity, minimize the emergence of resistant bacteria and can result in synergic interaction [11]. In current study, we tried to evaluate the effectiveness of rosemary essential oil alone and in combination with some antibiotics against biofilm of *K. pneumoniae* isolated from urine.

MATERIALS and METHODS

Bacterial isolates

Fourteen isolates of *K. pneumoniae* were obtained from Al-Yarmok hospital, Baghdad, Iraq. The isolates were isolated from urine and grown on MacCongy agar at 37ºC then identified using API-20E test kit (Bio-Mereix, France) in central health laboratory in Baghdad, Iraq according to the manufacture instructions.

Preparation of Essential Oil of R. officinalis

The air dried leaves (250 gm) of *R. officinalis* were used for isolation of essential oil by cleavenger hydrodistillation method. The distilled water (1.2 L) was added to dried material then boiled for 3 h, the essential oil was kept at 4ºC until use [12].

Antimicrobial sensitivity disc test

The antibiotics resistance pattern in *K. pneumoniae* isolates was detected by disc diffusion method on Mueller-Hinton agar (Himedia, India) using ceftriaxone (30 mg), cefotaxime (10 mg), cefodiziime (30 mg), cephalexin (30 mg) ampicillin (10 mg), aztreonam (30 mg), cefepime (30 mg), imipenem (10 mg), pipracillin (100 mg).

Determination of minimal inhibitory concentration (MIC)

The MIC values for different antibiotics and Rosemary E.O were determined by broth microdilution method in 96 well microtiter plate according to the clinical and laboratory standard institute [13].

Gentamicin (40 mg/ml), ciprofloxacin (500 mg dissolved in 0.1N HCl), amoxicillin (500 mg dissolved in distilled water), trimethoprim/sulfamethoxazole (80 mg dissolved in 0.1N HCl), cefotoxime (1000 mg dissolved in distilled water) and essential oil (10^5 µg/ml dissolving in dimethyl sulfoxide, DMSO) were used to prepared two fold serial dilutions with nutrient broth (Himedia, India) in a final concentration ranging from 256-2, 16-0.125, 2048-16, 512-16 and 2×10^4-156 µg/ml respectively for antibiotics and essential oil.

The bacterial inoculums was added at final concentration 10^6 cell/ml , bacteria and media control were prepared. The results were registered after incubation at 37ºC for 18 h by determining MIC (the lowest concentration of antibiotics and E.O that prevents growth of the microorganism). MIC was calculated by take the absorbance of the tested wells that did not exceed the value of the absorbance media control [14].

Biofilm formation assay

Biofilm was carried out in microtitere plate as described by ElFartas-Aissani *et al.* [15] with some modification. Briefly, *K. pneumoniae* isolates were grown in nutrient broth over night at 37ºC. 200 µl of bacterial suspensions ($O.D_{540}$ 0.64= 10^8 cell/ml) were transported to each well in triplicate and incubated 24 h at 37ºC and negative control prepared from media only. The media was removed and washed three times with phosphate buffer saline then 200 µl of crystal violate (0.1% w/v) added to the wells for 15 min at room temperature. Crystal violate was removed and washed three times with distilled water 200 µl of absolute ethanol (96%) (Merk, Germany) used to dissolve the crystal violate inside the cells and the absorbance was measured by an ELISA reader. Comparing the optical density (OD) of biofilm to the control and according to the reading the isolates were classified as follows: $OD \le OD_c$ no biofilm producer, $OD_c < OD \le 2\times OD_c$ weak biofilm, $2\times OD_c < O.D \le 4\times OD_c$ moderate and $4\times OD_c < OD$ strong biofilm.

Biofilm inhibition assay

The antibiofilm activity of used antibiotics and Rosemary E.O were tested on isolates of *K. pneumoniae* using 96 well microtiter plate as described by Selim *et al*. (2014) with modifications. Biofilm formation was achieved by adding 100 µl of bacterial suspension (10^8cell/ml) then incubated at 37 ºC for 24 h. The MIC and 1/2 MIC of antibiotics and Rosemary E.O were added to the wells alone and in combination. The medium without antibiotic or extract was used as control and the plate was incubated further at 37 ºC for 24 h. Following incubation, the crystal violet assay was performed as mentioned above. The reduction percentage in biofilm formation was measured using the formula [16]:

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

Statistical analysis

Results were analysed statistically by using ANOVA one way to compare means of MIC for antibiotic used and also for comparison between reduction percentage in biofilm due to used combinations of different antibiotics used and rosemary essential oil while Student's t-test was used for comparing between MIC and 1/2 MIC used alone and in combination. Differences were considered statistically significant if P-values were less than 0.05. All data were analyzed using the SPSS IBM version 20

RESULTS

The resistance parterres for 14 isolates of *K. pneumoniae* were determined for nine antibiotics using disc diffusion method (**Table 1**). After, the MICs of different antibiotics used and rosemary essential oil against *K. pneumoniae* were examined by broth microdilution method. The mean of MIC values were 128, 0.25, 768, 64, 384 and 10^4 µg/ml for gentamicin, ciprofloxacin, amoxicillin, trimethoprim/ sulfamethoxazole, cefotoxime and rosemary essential oil. According to the results, all *K. pneumonia*e isolates have ability to produce biofilm (100%) and classified as 5 (36%) isolates were strong biofilm producer and 9 (64%) isolates were moderate biofilm producers.

Table 1. Antibiotic resistance patterns of *K. pneumoniae* isolates. AM, ampicillin; ATM, aztreonam; CDZ, cefodiziime; CRO, ceftriaxone; CTX, cefotaxime; CL, cephalexin; FEP, cefepime; IPE, imipenem; PRL, pipracillin.

Isolates No.	Antibiotics resistance patterns								
K1	ATM	FEP	PRL	IPE	Am	CRO	CTX	CDZ	-
K2	ATM	FEP	PRL	IPE	Am	CRO	CTX	CDZ	CL
K3	ATM	FEP	PRL	IPE	Am	CRO	CTX	-	CL
K4	ATM	-	PRL	-	Am	-	-	CDZ	-
K5	ATM	FEP	PRL	IPE	Am	CRO	CTX	CDZ	CL
K6	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	-
K7	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	CL
K8	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	-
K9	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	-
K10	ATM	FEP	PRL	IPE	Am	CRO	CTX	CDZ	CL
K11	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	CL
K12	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	-
K13	ATM	FEP	PRL	IPE	Am	CRO	CTX	CDZ	-
K14	ATM	FEP	PRL	-	Am	CRO	CTX	CDZ	-

In addition, the results showed various percentage of reduction in biofilm formation *K. pneumonia*e isolates when treated with different concentration of antibiotics and rosemary essential oil (Fig 1).

When study the effect of MIC and 1/2 MIC of antibiotics and rosemary essential oil that used alone and in combinations found significant differences ($P \leq 0.05$) in biofilm reduction percentage of all isolates in comparison to control. In strong biofilm formation, the highest percentage of reduction was observed when used 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 were reduced from 0.36 to 0.18 and 0.36 to 0.175. While the highest percentage of reduction in moderate biofilm formation was showed when used combination of MIC of ciprofloxacin and 1/2 MIC rosemary essential oil (61%) that biofilm formation was reduced from 0.3 to 0.11.

The 1/2 MIC noticed significant differences ($P \leq 0.05$) for all antibiotics and essential oil in contrast with other combinations as well as reduced the biofilm formation 0.2-1.4 fold in contrast to MIC.

The type of antibiotics used in this study not demonstrated any statistical significant in biofilm reduction percentage as well as between moderate or strong biofilm producer isolates.

DISCUSSION

Infections associated with biofilm formation are very difficult to treat because the ability to resist antibiotic treatment due to less penetration of drug inside the biofilm in the presence of extracellular substance. The ability of biofilm formation in this study agrees with previous study [15]. The high rate of biofilm formation might be related to phenotypes and genes involved in biofilm such as capsule, lipopolysaccharide (LPS) and fimbriae [2]. According to disc diffusion method, the all studied *K. pneumoniae* isolates resist to different antibiotics and owing to the fact that multiple drug resistant is increasing in worldwide, thus, there is a growing need for other strategies for eradicating this pathogen and biofilm formation.

Several classes of antibiotics were used for medication of infections with *K. pneumoniae* such as cephalosporins and trimethoprim-sulfamethoxazole that are used in treating urinary tract infection (UTI) as well as carbapenems that choice for treating severe infections caused by the extended spectrum beta lactamase producing isolates [17], aminoglycosides and fluoroquinolones were used widely for limiting the growth of members of enterobacteriaceae family [8]. However, antibiotics might kill the free floating bacteria but fail to eradicate bacterial cells embedded in a biofilm. The MIC values for different antibiotics used in this study found to be effective when tested on planktonic bacteria but give low percentage of biofilm reduction when tested on biofilm formation because the minimal inhibitory concentration and minimal bactericidal concentration of antibiotics to bacteria growing in biofim may be 100-1000 fold higher as compared with planktonic bacteria [18]. Rosemary essential oil showed antimicrobial activity against different microorganisms especially multidrug resistant bacteria [19-21], in our study we found that the effective and enhance the activity of antibiotics against biofilm formation.

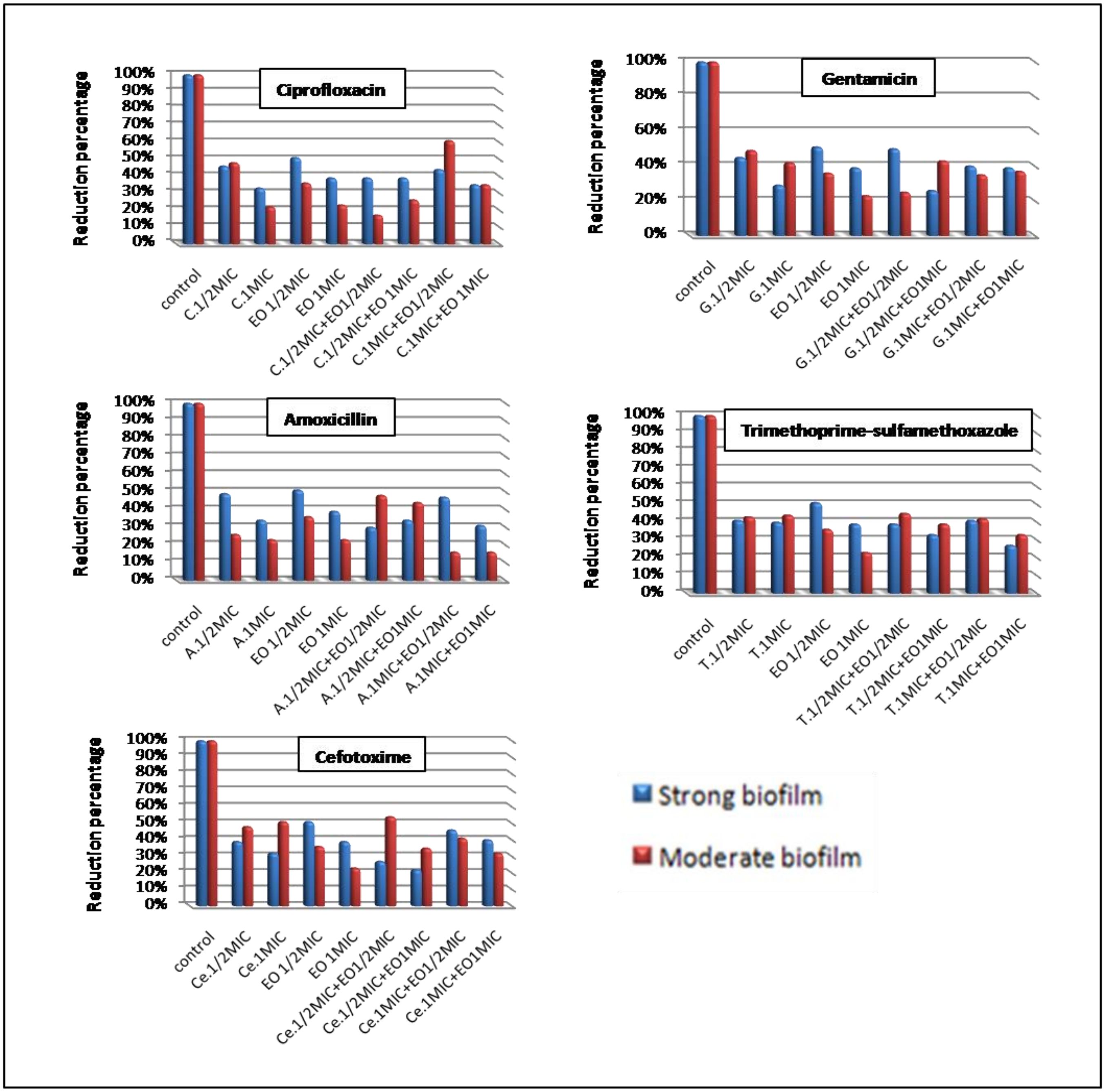

Fig 1. Reduction percentage of *K. pneumoniae* biofilm formation by different antibiotics and rosemary essential oil.

The effect of sub-inhibitory concentration (1/2 MIC) of antibiotics and rosemary essential oil on *K. pneumoniae isolated* observed high percentage of biofilm reduction. In previous studies, 1/10 MIC of gentamicin led to a reduction (0.2-0.9 fold) in biofilm formation of half *K. pneumoniae* isolates [22], Majta´n *et al.* [23] found that biofilm production of *Salmonella enteric* serovar Typhimurium was significantly lower after growth with 1⁄2 MIC of gentamicin. Moreover, the presence of 1/8 MIC of *Plectranthus amboinicus* essential oil caused an alteration of *K. pneumoniae* morphology besides reduction of capsule expression. When higher subinhibitory concentrations (1/4 and 1/2) of the essential oil were tested, almost no bacteria could be visualized, but only cellular debris [24]. Cephalosporin at a concentration 1/10 MIC caused morphological alterations, reduced in enterochelin and capsular materials production in strain of *K. pneumoniae* [9]. Alyasiri [25] was reported that the growth of *K. pneumoniae* in the presence of sub-MICs of ciprofloxacine reduced the viscous slime formation at 4 and 8 µg/ml and remarkably increased the amount of exopolysaccharide at low concentrations (2 µg/ml). The sub-inhibitory concent-rations of antibiotics have been shown *in vitro* effect to alter the ultrastructure and antigenicity of bacteria as well as their adherence to epithelial cells [26], furthermore, the subinhibitory concentrations of an essential oil can modify the architecture of bacterial surface and may interfere with some their functions [27].

This high resistance to antibiotics in biofilms back to reduced drug penetration into the deep layer of biofilm due to the presence of a physical barrier that is formed by the extracellular polymeric substance like capsule [22, 23].

Thus, the combination of antibiotics with rosemary essential oil could enhance the reduction of biofilm formation especially when these combination contain 1/2 MIC of antibiotic and/or 1/2 MIC of rosemary essential oil. This may be belonged to the hydrophobicity of essential oil was responsible for the disruption of bacterial structures that leads to increased permeability and rosemary essential oil was also able to inhibit quorum sensing [28]. From our previous work found that rosemary essential oil had antibacterial and antibiofilm activity against *K. pneumoniae* as well as affected the type 1 fimbriae at gene level probably by mutation during initial attachment of biofilm formation [20].

The essential oils have strong synergistic effects when combined with less effective antibiotics. Van Vuuren *et al.* [29] showed the combination between rosemary essential oil and ciprofloxacin had synergistic pattern against *K. pneumoniae* and the antagonistic, synergistic and additive interactions depending on the combined ratio. Combination of clove and rosemary essential oil with anti-pseudomonal drugs like ceftazidime, imipenem, aztreonam and ciprofloxacin could enhance the activity of theses drug [30].

The essential oil of *C. cyminum* decreased biofilm formation and enhanced the activity of the ciprofloxacin disk against *K. pneumoniae due to the ability of this E.O to* suppress of capsule expression and inhibit of urease activity of bacteria in biofilm *[31].*

In our results, the low MIC of ciprofloxacin (0.25 µg/ml) and high percentage of biofilm reduction (61%) when used combination of ciprofloxacin and rosemary essential oil could be attributed to the ability of ciprofloxacin to penetrate a biofilm due to not deactivate in the surface layers of the biofilm where as penicillin deactivate faster than it could diffuse in [32].

In spite of combination between antibiotics and rosemary essential oil for reduction of biofilm formation, it was still not eradicated. This due to resistance to antibiotics was not the only factor for biofilm resistance, but also reduced growth rate of the bacteria as well as changes in bacterial gene expression in the biofilm mode of growth were contributed to biofilm resistance [33].

Based on our findings, the rosemary essential oil showed good results when combination with antibiotics used for treatment of UTI and further studies of other subinhibitory combinations, including *in vivo* studies are required to assess the potential utility of these combinations to treat *K. pneumoniae* infections that resistant to antibiotics.

Conflict of interest

The authors declare that they have no conflict of interests.

REFERENCE

1. **Podschun R, Ullmann U.** (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**: 589-603.
2. **Vuotto C, Longo F, Balice M, Donelli G, Varaldo P.** (2014) Antibiotic resistance related to biofilm formation in *Klebsiella pneumoniae*. *Pathogens* **3**: 743-758.
3. **Bansal S, Harjai K, Chhibber, S.** (2015) *Aeromonas punctata* derived depolymerase improves susceptibility of *Klebsiella pneumoniae* biofilm to gentamicin. *BMC Microbiology* **15**:119.
4. **Jagnow J, Clegg S.** (2003) *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiol* **149**: 2397-2405.
5. **Di Martino P, Cafferini N, Joly B, Darfeuille-Michaud A.** (2003) *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. *Res Microbiol* **154**: 9-16.
6. **Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S** (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**: 860-864.
7. **Bansal S, Chhibber S.** (2010) Curcumin alone and in combination with augmentin protects against pulmonary inflammation and acute lung injury generated during *Klebsiella pneumoniae* B5055-induced lung infection in BALB/c mice. *J Med Microbiol* **59**: 429-437.
8. **Stewart PS, Costerton, JW.** (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358**:135-138.
9. **Kadurgamuwa JL, Anwar H, Brown M, Zak O.** (1985) Effect of subinhibitory concentrations of cephalosporins on surface properties and siderophore production in iron-depleted *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **27**: 220-223.
10. **Hussain A, Anwar F, Chatha A, Jabbar A, Mahboob S, Nigam P.** (2010) *Rosmarinus officinalis* essential oil: antiproliferative, antioxidant and antibacterial activities. *Braz J Microbiol* **41**: 1070-1078.
11. **Dawis MA, Isenberg HD, France KA, Jenkins SG.** (2003) In vitro activity of gatifloxacin alone and in combination with cefepime, meropenem, piperacillin and gentamicin against multidrug-resistant organisms. *J Antimicrob Chemother* **51**:1203-1211.
12. **Jamshidi R, Afzali Z, Afzali D.** (2009) Chemical composition of hydrodistillation essential oil of rosemary in different origins in Iran and comparison with other countries. *American-Eurasian J Agric Environ Sci* **5**: 78-81.
13. **Clinical and Laboratory Standards Institute (CLSI).** (2012) Microdilution method. Methods and Dilution Antimicrobial Susuptibility test for Bacteria that Grow Aerobically. Vol **32**(2). 9th ed. USA: 16-18.
14. **Budzynska A, Wieckowska-Szakiel M, Sadowska B, Kalemba D, Rozalska, B.** (2011) Antibiofilm activity of selected essential oils and their major components. *Polish J Microbiol* **60**: 35-41.
15. **El Fertas Aissani R, Messai Y, Alouache S, Bakour R.** (2013) Virulance profiles and antibiotics suscepitibility patterns of *Klebsiella pneumoniae* strains isolated from different clinical specimens. *Pathologie Biologie* **61**:209-216.
16. **Mathur S, Gutte M, Paul D, Udgire M.** (2013) Study the effect of essential oils on microbial biofilm formation by *Klebsiella pneumoniae*. *Sch Acad J Biosci* **1**:76-79.
17. **Liu H, Lin H, Lin Y, Yu S, Wu W, Lee Y.** (2011) Antimicrobial susceptibilities of urinary extended-spectrum beta-lactamase-producing Escherichia coli and *Klebsiella pneumoniae* to fosfomycin and nitrofurantoin in a teaching hospital in Taiwan. *J Microbiol Immunol infect* **44**:364-368.
18. **Hoibya N, Bjarnsholt T, Givskov M, Molin S, Ciofu O.** (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrobl Agents* **35**: 322-332.
19. **Najem AM, Abed IJ, AL-Haidari AM.** (2016) Evaluation the activity of rosemary (*Rosmarinus officinalis* L.) Essential Oil against Some Cyanobacteria. *Iraqi J Biotechnol* **15**:97-102.
20. **Abdulhasan GA.** (2015) The biological effect of *Rosmarinus officinelis* L. essential oil on biofilm formation and some fimbrial genes (*fimH-1* and *mrkD*) of *Klebseilla pneumoniae*. *Iraqi J Sci* **56**:2553-2560.
21. **Abdullah BH, Hatem SF, Jumaa W. (2015) A Comparative Study of the Antibacterial Activity of Clove and Rosemary Essential Oils on Multidrug Resistant Bacteria. *UK J Pharm Biosci* 3: 8-22.**
22. **Naparstek L., Carmeli Y, Navon-Venezia S, Banin E.** (2014) Biofilm formation and susceptibility to gentamicin and colistin of extremely drug-resistant KPC-producing *Klebsiella pneumoniae*. *J Antimicrob Chemotherapy* **69**: 1027-1034.
23. **Majta´n J, Majta´nova L, Xu M, Majta´n V.** (2007) In vitro effect of subinhibitory concentrations of antibiotics on biofilm formation by clinical strains of *Salmonella enterica* serovar Typhimurium isolated in Slovakia. *J Appl Microbiol* **104**: 1364-5072.
24. **Goncalves TB, Bragaa AG, de Oliveiraa F, Santiago GM, Carvalho CB, et al.** (2012) Effect of subinihibitory and inhibitory concentrations of

Plectranthus amboinicus (Lour.) Spreng essential oil on *Klebsiella pneumoniae*. *Phytomedicine* **19**: 962- 968.

25. **Alyasiri NS.** (2005) Effect of subinhibitory concentration and crude exopolysaccharide production by *klebsiella pneumoniae*. *Al-Taqani J* **18**: 121-127.
26. **Wojnicz D, Jankowski S.** (2007) Effects of subinhibitory concentrations of amikacin and ciprofloxacin on the hydrophobicity and adherence to epithelial cells of uropathogenic *Escherichia coli* strains. *Int J Antimicrob Agents* **29**:700-704.
27. **Derakhshan S, Sattari M, Bigdeli M.** (2008) Effect of subinhibitory concentrations of cumin (*Cuminum cyminum* L.) seed essential oil and alcoholic extract on the morphology, capsule expression and urease activity of *Klebsiella pneumoniae*. *Int J Antimicrob Agents* **32**:432-436.
28. **Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V.** (2013) Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* **6**:1451-1474.
29. **Van Vuuren SF, Suliman S, Viljoen AM.** (2009) The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. *Lett Appl Microbiol* **48**:440-446.
30. **El Hosseiny L, El-Shenawy L.** (2015) In vitro evaluation of the combination between some antibiotics and essential oils of clove and rosemary against *Pseudomonas aeruginosa*. *Antimicrob Resist Infect Control* **4:** 169.
31. **Derakhshan SSM, Bigdeli M.** *(2010) Effect of cumin (Cuminum cyminum) seed essential oil on biofilm formation and plasmid Integrity of Klebsiella pneumoniae. Pharmacogn Mag* ***6****: 57-61.*
32. **Anderl J, Franklin M, Stewar P** (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* **44**: 1818-1824.
33. **Anderl JN, Zahller J, Roe F.** (2003) Role of nutrient limitation and stationary-phase existence in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* **47**:1251-1256.

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