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Effects of Phenolic Plant Extracts on Biofilm Formation by *Klebsiella pneumoniae* Isolated from Urinary Tract Infections

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Abstract

Ten isolates of *Klebsiella pneumoniae*, seven isolates of *Pseudomonas aeruginosa* and nine isolates of *Staphylococcus aureus*, were obtained from 100 urine samples collected from Baghdad hospitals. All isolates were identified biochemically and confirmed by using VITEK 2 and were then tested for their susceptibility towards 6 antibiotics and for phenolic extracts of *Thymus vulgaris* and *Cinnamomum cassia*. All bacteria were greatly affected by *T. vulgaris*, especially *K. pneumoniae*. Viable count was performed, it was noted that the number of bacterial cells reduced from 1×10^8 CFU to 1.2×10^3, 2×10^5 and 1.8×10^6CFU of *K. pneumoniae*, *P. aeruginosa* and *S. aureus* respectively. While *C. cassia*had a slight effect on them. *K. pneumoniae* isolates which were affected by phenolic extract more than the other bacteria under study and at the same time were resistant to more than one type of tested antibiotics. These isolates were taken to detect their ability to form biofilm by using Congo red as screening method for it. The results showed that all isolates produced biofilms. Also, by using microtiter plate method, the results confirmed that all isolates produced biofilm where 7 isolates were strong biofilm producers and 3 were moderate. The strongest isolate was taken to study the effect of *T. vulgaris* and *C. cassia* phenolic extract on its biofilm formation by using microtiter plate method with two concentrations (20 and 40 ml/L). The results showed that biofilm reduction was 45% and 73% for *T. vulgaris* and that for *C. cassia*it was 15% and 20% after using 20 and 40 ml/L respectively.

Keywords: UTIs, *Klebsiella pneumoniae*, Phenolic plant extract, Biofilm

تأثيرالمستخلصات النباتية الفينولية في تكوين الأغشيةالحيوية بواسطة*Klebsiella pneumoniae* المعزولة من اصابات المسالك البولية

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الخلاصة

تم الحصول على عشر عزلات من*Klebsiella pneumoniae*و 7 عزلات من *Pseudomonas aeruginosa*و 9 عزلات من*Staphylococcus aureus* من 100عينة ادرار تم جمعها من مستشفيات بغداد. شخصت جميع العزلات بالاختبارات الكيميائية وتم تاكيد التشخيص باستخدام2 VITEK، تم اختبار مدى حساسية العزلات المذكورة انفا تجاه 6 مضادات حيوية والمستخلصات الفينولية من *Thymus vulgaris*

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و*Cinnamomumcassi*. تأثرت جميع عزلات البكتريا بشكل كبير بنبات الزعتر وخصوصا *K. pneumoniae*,واختزل عدد الخلايا البكتيرية من1×10^8وحدة مكونة للمستعمرة إلى (1.2×10^3و 2×10^5 و 1.8×10^6 وحدة مكونة للمستعمرة) لبكتريا*K. pneumoniae*و*P.aeruginosa* و *S. aureus*على التوالي ، بينما كان للقرفة*C. cassia* تأثير طفيف عليهم. لوحظ ان عزلات بكتريا *K.pneumoniae*تاثرت بمستخلص الفينول أكثر من باقي البكتيريا التي تمت دراستها وفي نفس الوقت كانت مقاومة لأكثر من نوع واحد من المضادات الحيوية المختبرة ، اختيرت هذه العزلات لدراسة قابليتها على تكوين الأغشية الحيوية الخاصة بهم باستخدام الكونغو رید وأظهرت النتائج أن جميع العزلات كانت منتجة للغشاء الحيوي.كذلك باستخدام لوحة المايكروتايتر، وقد اظهرت النتائج ان 7 من العزلات كانت عالية بانتاج الغشاء الحيوي و3 متوسطة الانتاج. اختيرت أقوى العزلات لدراسة تاثيرالمستخلصات الفينوليةلنباتي الزعتر *T. Vulgaris*و القرفة *C. cassia*على تشكيل غشائها الحيوي باستخدام طريقة لوحة ميكروتيتر بتركيزين (20 و 40 مل/لتر). اظهرت النتائج ان اختزال الغشاء الحيوي كان 45٪ و 73٪ بنبات الزعتر وكذلك 15٪ و20 ٪ بنبات القرفة بعد استخدام 20 و 40 مل/لتر على التوالي.

الكلمات المفتاحية: اصابات الجهاز البولي, كليبسيلا الرئوية ، المستخلصات النباتية الفينولية ، الغشاء الحيوي

Introduction

Klebsiella is a gram negative, non-motile, oxidase-negative, rod shaped bacteria with a prominent polysaccharide-based capsule[1]. *Klebsiella* species are found everywhere in nature. This is thought to be due to distinct sub lineages developing specific niche adaptations, with associated biochemical adaptations which make them better suited to a particular environment. They can be found in water, soil, plants, insects and other animals including humans[2][3]. *Klebsiella* species that is routinely found in the human nose, mouth, and gastrointestinal tract as normal flora, can also behave as opportunistic pathogens[4].This species is also known to infect a variety of other animals, both as normal flora and opportunistic pathogens[2]. *Klebsiella* organisms are resistant to multiple antibiotics. This is thought to be a plasmid-mediated property. Length of hospital stay and performance of invasive procedures are risk factors for acquisition of these strains[5].

Although some *Klebsiella* infections can be effectively treated with single-agent therapy involving penicillin or a similar antibiotic, the emergence of organisms that are resistant to these drugs, has necessitated the development of novel therapeutic approaches[6],*K. pneumoniae* is resistant to beta-lactam antibiotics, a group that includes carbapenems, penicillin, and cephalosporin[7].As a result, drug-resistant *K. pneumoniae* infections typically require combination therapy with structurally diverse agents, such as a beta-lactam antibiotic and an aminoglycoside[8].The urinary tract is the most common site of infection. *Klebsiella* accounts for 6 to 17% of all nosocomial urinary tract infections (UTIs) and shows an even higher incidence in specific groups of patients at risk, e.g., patients with neuropathic bladders or with diabetes mellitus [9].As a cause of nosocomial gram-negative bacteremia, *Klebsiella* is second only to *Escherichia coli*[10]. Hospital-acquired bacterial infections caused by *Klebsiella* spp., including *K. pneumonia*e are among the most frequently recovered etiologic agents from nosocomial infections [11].This opportunistic pathogen can generate a thick layer of biofilm as one of its important virulence factors, enabling the bacteria to attach to living or abiotic surfaces which contributes to drug resistance [12].

Biofilms protect microorganisms from opsonization by antibodies, phagocytosis and removal via the ciliary action of epithelial cells [13].In addition, bacterial populations in biofilms are considerably more resistant than free-living planktonic cells are to antibacterial

agents. Therefore, treatment of an infection after a biofilm has been established is frequently futile with the current therapy options [11].

With the lack of effective antibiotics rapidly becoming a global issue, research into sourcing potential new antimicrobials from plant products has gained immense importance. Thymol, the most active ingredient in the herb, has strong anti-bacterial properties and expectorant effect. In addition to thymol, thyme contains the flavonoids luteolin, apigenin, thymonin and naringenin which are antioxidants. Thyme can be used as disinfectant, and it has antiseptic and antifungal properties [14].

There are several types of cinnamon which are significant, well-liked spices employed across the world, not just in cooking but also in conventional and current therapies [15].The leaves and barks have antibacterial properties and are frequently used to cure a variety of illnesses [16].

Many studies have found the effectiveness of *T.vulgaris* and *C.cassia* against different types of pathogenic bacteria, especially their essential oils, although the effect of their phenolic extracts has been much less. Hence the aim of this study was to investigate the antibacterial and antibiofilm effects of these extracts.

Materials and Methods

Laboratory Prepared Culture Media

All ready-media were prepared as recommended by manufacturing company instructions. The constituents after being dissolved in distilled water, were sterilized by autoclaving at 121ºC/15 pound/inch^2 for 15 min and then dispensed into sterile petri dish or a tube as required to be checked for sterility and then stored at 4ºC until used [17].

Congo red agar it is not ready-media, but is rather prepared by dissolving 50g of sucrose,15g agar-agar and 37g of ready-media brain-heart infusion broth in 900ml of D.W. and then sterilized by autoclaving at 121ºC/15 pound/inch^2 for 15 min, waiting until cooled to 55ºC. Congo red dye is prepared as 0.8g of the dye dissolved in 100 ml of D.W. and added to that medium aseptically, later dispensed into sterile petri dish or tube as required [18].

Urine samples (n:100) were collected from patients with UTIs in hospital in Baghdad. The samples were cultured on MacConkey agar and mannitol salt agar and detected by VITEK2 compact system [19].The bacterial isolates were tested for their capability to form biofilm and the effects of phenolic plant extract on them. Also, the antibiotic susceptibility test was applied on bacterial isolates.

Antibiotic Susceptibility Test by Disc Diffusion Test

The modified Kirby-Bauer method (disc diffusion test) was used to test the susceptibility of bacterial isolates to amikacin, azithromycin, erythromycin, ceftazidime, vancomycin and ciprofloxacin as the following:

Preparation of Mueller-Hinton Plates

Mueller-Hinton agar plates were prepared according to the manufacturer's instructions and then the medium was cooled to 45-50ºC and poured into the plates which was then allowed to set on a level surface to a depth of approximately 4mm. The plates were stored at 4 ºC until use after the agar had solidified [20].

Inoculum Preparation

To prepare the inoculum, colonies of all bacterial isolates from overnight culture were transferred to 5 ml tube of normal saline to obtain culture with 1×10^8 CFU/ml by adjusting to 0.5 McFarland tube.

Inoculation of the test plate

A. The plates were inoculated by dipping a sterile swab into the inoculums. Care must be taken to express excess broth from the swab prior to inoculation, by pressing and rotating the swab firmly against the side of the tube above the level of the fluid.

B. Swab was rubbed over the surface of the medium three times rotating the plate through at an angle of 60 after each application. Finally, the swab was passed around the edge of agar surface.

C. Inoculums were left for a few minutes to dry at room temperature with closed lid.
D. Antibiotic discs were placed on the inoculated plate by using a sterile forceps. Discs should be warmed to room temperature, and then dispensed on the agar surface and pressed down gently with sterile forceps.

E. The plates were incubated within 30 min. for 18-24 hrs. at 37ºC.

Reading the Results

After incubation, the diameter of each inhibition zone was measured by millimetre using ruler. The isolate was interpreted as either susceptible, intermediate, or resistant to a particular drug by comparison with standards inhibition zone.

Determination the Effect of Phenolic Plant Extracts on Bacterial Isolates

The plants *T. vulgaris* and *C. cassia* were obtained from the local markets and phenols were extracted according to the method laid down by Arciola *et al*.[21]. Stock solutions of phenolic plant extracts of *T. vulgaris* and *C. cassia* were prepared, two concentrations, 20ml/L and 40ml/L, were prepared by mixing nutrient agar (V: V) with the phenolic extract for each plant (*T. vulgaris* and *C. cassia*), and then poured in sterilized petri dishes [20].Viable count was performed to detect the phenolic extract effects on bacterial isolates.

Detection of Biofilm FormationbyCongo-red Agar Method

Inoculation Preparation: to prepare the inoculum, colonies from overnight culture of bacteria were transferred to 5 ml tube of normal saline to obtain culture with 1×10^8 CFU/ml by adjusting to 0.5 McFarland tube. Inoculation of the Plate: the streaking was done using an inoculation loop. The inoculation loop was first sterilized by passing it through a flame. When the loop became cool, it was dipped into an inoculum broth containing bacteria. The inoculation loop was then dragged across the surface of the agar back and forth in a zigzag motion until approximately 30% of the plate was covered. The loop then was re-sterilized, and the plate was turned 90 degrees. Starting from the previously streaked section, the loop was dragged through it two to three times continuing the zigzag pattern. The procedure was then repeated once more taking care not to touch the previously streaked sectors. In this procedure, each time the loop gathers fewer and fewer bacteria until it gathers just single bacterial cells that can grow into a colony. The plate should show the heaviest growth in the first section. The second section will have less growth and a few isolated colonies, while the last section should have the least amount of growth and many isolated colonies [22]..

Biofilm Formation Assay

Biofilm assay was performed in well microtiter plate according to Mindell [17] with some modification. Briefly. *K. pneumonae* isolates were sub cultured in brain heart infusion broth

(Himedia/India) over night at 37ºC. Two hundred µL of bacterial cultures (O. D_{540} 0.64=10^8 cell\ml) were transferred to each well in triplicate and then incubated 24hr at 37ºC 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 15min 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 (100µl) and the absorbance was measured by an ELISA reading. After comparing the optical density (O.D) of biofilm to the control and according to the reading, the isolates were classified as follows: O. D≤ $O.D_c$ no biofilm producer biofilm, Dc<O.D≤ 2 X O.D weak biofilm, 2 X O. Dc<O.D≤4 XO. D moderate biofilm and 4 X O. Dc< O.D strong biofilm [17].

Biofilm Inhibition Assay

The anti-biofilm activity of phenolic extracts was tested onbacterial isolate (strongest biofilm producer) using 96 well microtiter plate as described by Selim *et.al*. [23] with modifications. Biofilm formation was achieved by adding 100ml of bacterial suspension (10^8cell\ml) and then incubated at 37ºC for 24hr. Two concentrations of plant extracts were added in the wells. The medium without extract was used as control and the plate was incubated further at 37ºCfor 24hr. Following incubation, the crystal violet assay was performed as mentioned above. The percentage reduction in biofilm formation was measured using the following formula [22]:

$$Reduction\% = \frac{\text{Control O. D} - \text{Test sample O. D}}{\text{Control O. D}} X100$$

Results and Discussion

Identification of Bacterial Isolates

After culturing urine samples on MacConkey agar, mannitol salt agar and cetrimide agar, *K.pneumoniae*, *S.aureus* and *P.aeruginosa* were isolated and confirmed by using VITEK2.Ten isolates of *K. pneumoniae,* seven isolates of *P. aeruginosa* and nine isolates of *S. aureus* were obtained. All bacterial isolates were tested for their susceptibility to antibiotics. Results are shown in Table 1.

Table1: Resistance percentages of *K. pneumonia, S. aureus* and *P.aeruginosa* towards antibiotics.

Antibiotics	*K. Pneumoniae*	*S. aureus*	*P.aeruginosa*
Amikacin	90% s	90%	90%
Azithromycin	60%	50%	50%
Erythromycin	0%	20%	30%
Ceftazidime	0%	40%	0%
Vancomycin	0%	0%	0%
Ciprofloxacin	90%	90%	90%

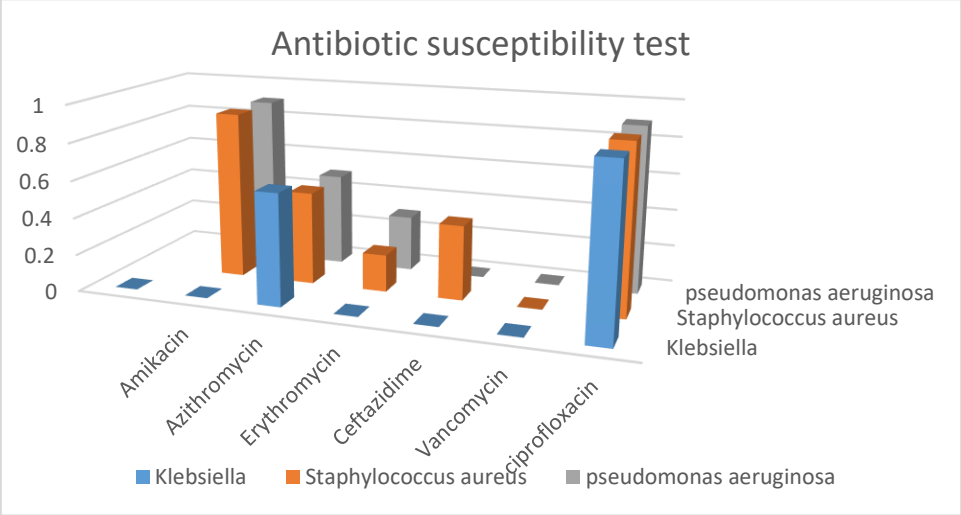

Figure 1: Resistance percentages of *K. pneumonia, S. aureus* and *P.aeruginosa* towards antibiotics.

The results showed that all bacterial isolates were resistant to vancomycin, but the best antibiotic is amikacin which was effective against them all.
The development of drug-resistant pathogens in patients with serious infections such as UTIs, has generally been ascribed to the widespread use of antimicrobial agents and the limited availability of infection prevention and control programs. As a result, it is increasingly common to encounter individuals infected with bacterial pathogens that are resistant to almost all currently available antibiotics. Of particular concern in the healthcare setting is the emergence of resistant gram-negative pathogens, including ESBL-producing K. pneumonia. While antibiotic resistance was previously noted mainly in nosocomial UTIs, it is nowadays also frequently observed in community-acquired UTIs [10][11].

Determination the Effects of Phenolic Plant Extracts on Bacterial Isolates
Phenolic extract was performed on *T. vulgaris* and *C.cassia* and added to the nutrient agar medium (V:V) in percentage of40 ml/L. Viable count was performed and all bacteria were found to be greatly affected by *T. vulgaris* specially *K. pneumoniae.* The number of bacterial cells reduced from 1×10^{8}CFU (Control)to $1.2\times 10^{3,}$ 2×10^{5} and 1.8×10^{6} CFU for *K.pneumoniae, P. aeruginosa* and *S.aureus* respectively. Whereas *C.cassia* had slight effects on them and the number of bacterial cells reduced to 5×10^{5}, 1.5×10^{6}, 3×10^{6} CFU respectively (Figure 2, Table 2).

Figure 2: Effect of *T. vulgaris* phenolic extract (40 ml/L) on *K.pneumonia*e.

Table 2:Effect of *Thymus vulgaris* phenolic extract(40 ml/L) on *K.pneumoniae, P. aeruginosa* and *S. aureus* cell number.

Detection of Biofilm Formation

K.pneumoniae isolates which were affected by phenolic extract more than the other bacteria understudy and the same time were resistant to more than one type of tested antibiotics, were taken to study the effects of the *T. vulgaris* phenolic extract on their biofilm formation [24].

Biofilm formation of *K. pneumoniae* was detected by using Congo red as a screening method,Figure (3), and by using a microtiter plate method.The results showed that all isolates produced biofilm (7 isolates were strong biofilm producers and 3 were moderate).

Figure 3: *K.pneumoniae* appears asblack colonies with a dry crystalline consistency indicated biofilm productionon Congo-red agar.

K.pneumoniae Biofilm Inhibition Assay by Plant Extract

The strongest isolate K1 was taken to study the effect of *T. vulgaris* and *C.cassia* phenolic extract on its biofilm formation by using microtiter plate method with two treatments (20 and 40 ml/L). The results showed that biofilm reduction was 45% and 73% for *T. vulgaris* and 15%and 20% for *C.cassia* after using 20ml/L and 40ml/L respectively (Table3).

Table 3: *K.pneumoniae* biofilm inhibition by plants phenolic extracts.

Plant Phenolic Extract	Conc. 20 ml/L	Conc. 40 ml/L
T.vulgaris	45%	73%
C.cassia	15%	20%

Solvents play a vital role in extraction of plant components. It has been found that the plants in various solvents exhibit different activity against the PAO1 biofilm. Comparatively, 1% methanolic extractof *Bergenia ciliata* (rhizome with skin) showed more than 80% inhibition of biofilm formation [24].

Syzygium legatii, *Syzygium masukuense*, and *Syzygium* speciesA had the best activities against gram-negative and-positive bacteria. The plant extracts had a good capacity to reduce biofilm formation and good to poor potential todestroy pre-formed biofilms [25].

Mohsenipour and Hassanshahian reported that *T.vulgaris* extracts can be useful as antimicrobial agents towards the pathogenic bacteria particularly in biofilm forms [26].

The results ofsome other studies that used *T.vulgaris* and *C. verum* essential oils against biofilm formation showed that cinnamon essential oil was more effective than thyme essential oil [27][28].

Firmino *et.al.* reported that *C.zeylanicum* and *C. cassia* essential oils as well as cinnamaldehyde were active against *Streptococcus pyogenes*, *P. aeruginosa*, and *Escherichia coli* biofilms in all of the concentrations and the substances analyzed biofilm biomasses were also reduced by up to 99.9 [29].

Most studies were about plants essential oils and their effects on pathogens. Only a few were about phenolic extracts. From our results, we concluded that phenolic extract of *T. vulgaris* is effective against *K.pneumoniae* isolates from urine, by reducing their numbers and biofilm formation, and that it can be used as an alternative to antibiotics to treat infections caused by this bacteria.

References

[1] N.S. Sheerin and E.K. Glover. "Urinary tract infection. Medicine". vol. 47, no. 9 , pp.546–550, 2019.

[2] M.P. Freire, L. Martinho, C.V. Mendes, F. Spad, F.J. De Paula, W.C. Nahas, E. David-Neto and L.C. Pierrotti. " Institutional protocol adherence in the incidence of recurrent urinary tract infection after kidney transplantation. " *Journal of Global Antimicrobial Resistance* 23 , pp.352–358, 2020.

[3] M.R. Asadi Karam,M.Habibi, S. Bouzari. "Urinary tract infection: pathogenicity, antibiotic resistance and development of effective vaccines against uropathogenic*Escherichia coli*. " *Molecular immunology* 108 , pp. 56–67, 2019.

[4] J.T. Matulay,C.M.Mlynarczyk and K.L. Cooper. Urinary tract infections in women: pathogenesis, diagnosis, and management". *Current Bladder Dysfunction Reports* 11 , pp. 53–60,2016.

[5] K.J. Ryan and C.G. Ray (editors). *Sherris Medical Microbiology* (4th ed.). McGraw Hill 2004 , p. 370.

[6] S. Brisse, F. Grimont and P.D.Grimont. *Prokaryotes*, New York, NY: Springer New York. 2006 pp. 159–196.

[7] - I.J. Adeosun, K.E. Oladipo , O.A. Ajibade, T.M. Olotu, A.A. Oladipo , E.H. Awoyelu, O.A. Alli , O.M. Oyawoye. "Antibiotic susceptibility of *Klebsiella pneumoniae* isolated from selected Tertiary Hospitals in Osun State, Nigeria". *Iraqi Journal of Science* , vol. 17 , pp. 1423-9 , 2019.

[8] S. R. M. Jadhav, G.Nageshawari, U. P. G.Mahadev, A. Kalpanai, V.Chanda. "INCREASING Incidence of multidrug resistance *klebsiella pneumoniae* infections in hospital and community settings". *International Journal of Microbiology Research*. vol. 4 n. 6 , pp. 253–257, 2012.

[9] R. Daza, J. Gutierrez, G. Piedrola. "Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections".*Internationaljournal of antimicrobial agents*, 18, pp. 211-215,2001.

[10] A. Bag,S.Bhattacharyya and R.Chattopadhyay. "Medicinal plants and urinary tract infections: an update". *Pharmacognosy Review* 2:277,2008.

[11] I A. Ibrahim . "Study the Effect of some Medical Plants in Biofilm Formation and Antibiotic Sensitivity for *Klebsiella Pneumoniae*". *Iraqi Journal of Science*. vol. 58, no. 2C, pp.971-83.2017 M.S. Bader, J. Hawboldt, A. Brooks. "Management of complicated urinary tract infections in the era of antimicrobial resistance" *Postgraduate Medicine*, vol. 122 no. 6, pp. 7-15,2010.

[12] J.W.Costerton , L. Montanaro , C.R. Arciola. "Biofilm in implant infections: its production and regulation". *International journal of Artificial Organs*, v.28 , no. 11 , pp.1062–8. 2005

[13] J.N. Anderl, M.J. Franklin, P.S. Stewart. " Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin". *Antimicrobial Agents Chemotherapy* . vol.44 no.7 , pp.1818–24, 2000.

[14] P.V. Rao, S.H. Gan, " Cinnamon: a multifaceted medicinal plant, " *Evidence-Based Complementary and Alternative Medicine.* , 642942, 2014 .

[15] A.D. Melo, A.F. Amaral, G. Schaefer, F.B. Luciano, C. de Andrade, L.B. Costa, M.H. Rostagno, "Antimicrobial effect against different bacterial strains and bacterial adaptation to essential oils used as feed additives", *Canadian journal of veterinary research*. v ol. 79 no.4 , 285–289, 2015.

[16] S.Ghafourian ; R Mohebi ; M . Rezaei ; M.Raftari; Z.Sekawi; H. Kazemian. "Comparative analysis of biofilm development among MRSA and MSSA strains"*Romanian Archives Microbiology Immunology* , vol. 71, no. 4pp. 175–82.2012

[17] E. Mindell .Earl Mindell's Herb Bible. New York. Simon & Schuster 2001.

[18] S. Bose; M. Khodke ; S. Basak ; S. K. Mallick. "Detection of biofilm producing Staphylococci: Need of the hour". *Journal of Clinical and Diagnostic Research*. vol.3:1915-1920, 2009.

[19] T.K.Ling, Z.K.Liu, A. F. Cheng. " Evaluation of the VITEK 2 system for rapid direct identification and susceptibility testing of gram-negative bacilli from positive blood cultures". *Journal of clinical microbiology*. vol. 41, no. 10, pp. 4705-7, 2003.

[20] J.Balch,. and P. Balch: Prescription for Nutritional Healing. New York. Avery Publishing Group 2006.

[21] C.R. Arciola; L. Baldassarri; L. Montanaro. "Presence of icaA and icaD genes and slime production in a collection of Staphylococcal strains from catheter associated infections". *Journal of clinical microbiology*, vol 39 , no. 6 , pp. 2151-2156, 2001.

[22] T.Mathur; S.Singhal ; S. Khan; D.J.Upadhyay, T.Fatma, A.Rattan. "Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods". *Indian Journal of Medical Microbiology* vol. 24 , no.1 pp.:25-29. 2006.

[23] S. Selim; A. Amin; S. Hassan; M. Hagazey."Antibacterial, cytotoxicity and anticoagulant activities from *Hypnea esperi* and *Caulerpa prolifera* marine algae". *Pakistan Journal of Pharmaceutical Sciences*, Vol. 28, no.2, pp.525-530, 2015.

[24] B. S.Fazly Bazzaz, S. Darvishi Fork, R. Ahmadi, B. Khameneh. " Deep insights into urinary tract infections and effective natural remedies". *African Journal of Urology*. vol. 27, no. 1, pp. :1-3, 2021

[25] H. Khelifi, D.A.Saada, A.M. Bekada, N. Dehimeche. "Production and quality assessment of a set-style yogurt fortified with low concentrations of Thymus vulgaris L phenolic extracts"*South Asian Journal Experimental Biology*,vol. 8 , no .6 , pp. 222-234, 2018.

[26] Z. Mohsenipour and M. Hassanshahian. " The inhibitory effect of Thymus vulgaris extracts on the planktonic form and biofilm structures of six human pathogenic bacteria". *Avicenna J Phytomed*.; vol. 5 no. 4, pp. 309-18, 2015.

[27] F.Noorbakhsh , P.Rahmati. "Effects of Thymus vulgaris and Cinnamomum verum Essential Oils on bap and ica Gene Expression in *Staphylococcus aureus*". *Archives of Clinical Infectious Diseases*.vol. 17, no.2 , 2022.

[28] E.G. Sweedan " Estimate Antimicrobial activity and Anti-biofilm formation of bark Cinnamomum zeylanicum on *Klebsiella pneumoniae* isolated from Urinary Tract Infections". *Iraqi Journal of Science*. vol. 25, pp. 1560-6. 2018.

[29] D.F. Firmino, Cavalcante TTA, Gomes GA, Firmino NCS, Rosa LD, de Carvalho MG, Catunda FEA Jr. " Antibacterial and Antibiofilm Activities of Cinnamomum Sp. Essential Oil and Cinnamaldehyde: Antimicrobial Activities". *ScientificWorldJournal*. vol. 6, pp. 7405736., 2018.