Antimicrobial activity of *Thymus vulgaris* Essential oil Against Multidrug-Resistant *Klebsiella pneumoniae* Carrying bla NDM gene

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Abstract

According to the World Health Organization, *Klebsiella pneumoniae* is an opportunistic pathogen that is responsible for a range of infections, including urinary tract infections (UTIs). This particular species is considered a high-priority concern due to the worldwide issue of antimicrobial resistance. Medicinal plants and their essential oils have always had a variety of uses for different purposes, such as treating diseases worldwide. Therefore, they have a high economic value. The essential oil of *Thymus vulgaris* was assessed as a replacement for antibiotic growth promoters in the diet of quail chicks and in vitro tests. The study aimed to investigate the antibacterial effects of *Thymus vulgaris* essential oils on multidrug-resistant *Klebsiella pneumoniae* clinically isolated, analyze the antibiotic resistance profile and clinical treatment, and the pathogenicity of the NDM-positive gene *Klebsiella pneumoniae*. A total of 127 clinical specimens believed to be *Klebsiella pneumoniae*, including sputum, blood, urine, burn, wound, and stool swabs, were obtained from both genders and different age groups. The samples were streaked on different agar, and the bacterial growth was identified by using biochemical tests and the Vitek®2 system to confirm it. The frequency of antibiotic susceptibility was determined using the Vitek®2 system.

Out of 127 clinical samples, *K. pneumoniae* was recorded in 50 (39%) cases. The highest isolation rate of *K. pneumoniae* was in urine 17 (34%). Furthermore, females were more affected by 32 (64%) with *K. pneumoniae* than males 18 (36%), The results showed that *Klebsiella pneumoniae* isolates were highly resistant to ampicillin (100%), and more sensitive to thymus vulgaris. Molecular detection of The carbapenemase virulence gene, bla NDM was recorded (32%, 16/50). The significant effect of thyme essential oil as an antibacterial agent against *K. pneumoniae* has made it a promising natural agent for combating microbial infections.

Keywords: *Klebsiella pneumoniae, Thymus vulgaris,* Multi-resistant Bacteria

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Introduction

Klebsiella pneumoniae (KP) is an encapsulated, gram-negative bacterium found in the environment (Chen et al., 2023). non-motile, facultatively anaerobic bacteria (Chang et al., 2021). belonging to the family Enterobacteriaceae (Li et al., 2022). widely prevalent in the human and animal respiratory tract and intestinal tract, not only in the natural, soil, and water. KP can easily colonize the surface of the human respiratory mucosal and gastrointestinal tract and cause bacteremia, liver abscesses, pneumonia, and UTIs in the clinic (Liang et al., 2022). Several virulence factors, including lipopolysaccharides, capsules, fimbrial adhesins, and siderophores, are thought to contribute to *K. Pneumoniae's* pathogenicity (Sunil Lobo et al., 2020). the increase of antimicrobial resistance between bacteria and associated infections is a growing problem and poses a serious threat to human health (Ghanbarinasab et al., 2023). The global public health community has been threatened by the spread of Enterobacteriaceae that produce carbapenemase. Traditionally employed to treat infections brought on *Escherichia coli* and *K. pneumoniae* that led to extended-spectrum β-lactamase, carbapenems are currently regarded as last-resort antibiotics (Han et al., 2020). Global cell adaptation (alterations in cell regulation), decreased permeability into the bacterial outer membrane, efflux pumps, target modifications, and antibiotic destruction or modification by specific enzymes are common mechanisms of antibacterial resistance (Christaki et al., 2020). There are three Ambler classes for carbapenemase: A, B, and D. The most prevalent of class B carbapenemase is (NDM) New Delhi metalo β-lactamase (Dalmolin et al., 2017). NDM is a recently described enzyme that confers resistance to all ß-lactams antibiotics, particularly carbapenems, except monobactams, among the newly identified metallo-β-lactamases. NDM in 2008, in New Delhi, India. its initial identification has been reported by different countries around the world as an important health concern (Firoozeh et al., 2017). Since bacteria are continuing to develop antibiotic resistance, more and more scientists worldwide are searching for novel and efficient techniques to fight pathogens and associated illnesses. Due to antibacterial medications, we can overcome many infections. However, a considerable threat is posed by strains that have developed resistance mechanisms to widely used antibiotics (Kwiatkowski et al., 2022). One of the most important species of the Lamiaceae family, *Thymus vulgaris*, is native to the Mediterranean region and is used worldwide as a seasoning in cooking and liquors, in addition to its therapeutic use (Diniz et al., 2023). There are 928 species of this plant, which is native to Europe and grow naturally in the Mediterranean basin and northern Europe. It can also be found in other regions across the globe, including Asia, South America, and Australia (Dosary, 2018). Chemical analyses have shown the presence of various compounds in the essential oil of *Thymus vulgaris*. These compounds include flavonoids, tannins, saponins, and phenols. Additionally, it is rich in the monoterpene thymol and its isomer, carvacrol. This medicinal plant can display antimicrobial, antifungal, antioxidant, antiviral, anti-cancer, and anti-inflammatory activities due to the existence of thymol and carvacrol (Dehghani et al., 2019). Therefore, This investigation aimed to examine the antibacterial effects of essential oils from *Thymus vulgaris* (TVE) on multidrug-resistant *K. pneumoniae* clinically isolated, Examine the profile of antibiotic resistance and the clinical treatments, and the pathogenicity of the bla NDM-positive gene *K. pneumoniae* was suggested.

Material and methods

Bacteria isolate preparation

A total of one hundred twenty-seven clinical specimens suspected to be *Klebsiella pneumoniae*, including sputum, blood, urine, burn, wound, and stool swabs, were obtained from both genders and different ages. These specimens have been collected from different hospitals (Al-Nasiriyah Teaching Hospital, Al-Hussein Teaching Hospital, Al-Musawi Children's Hospital, Al Haboubi Hospital, and Bint Al Huda Hospital) in Thi-Qar Governorate, Iraq during the period from 1/3/2023 to 1/8/2023.

Identification of K. pneumoniae using the VITEK2 system

To identify the bacteria using 22 various types of antibiotics, a Vitek2 compact system with the AST–N326, AST–N327 (BioMérieux–France), and GN–6 (BioMérieux–France) Gram-negative bacteria detection kit was used. After that, confirmation of the *K. pneumoniae* isolates was performed (Ibrahim et al., 2022).

Antimicrobial susceptibility test

The VITEK-2 compact system was used to determine antibiotic susceptibility against various antimicrobial agents through antimicrobial susceptibility testing cards, concurrently with the identification of the bacterial genus using this method. In the VITEK-2 system, susceptibility to various antibiotics was interpreted according to CLSI guidelines (2023) for Enterobacteriaceae (Muhsin et al., 2022). The antibiotics applied were as follows. AMP: ampicillin (10 μg), PIP: piperacillin (10 μg), ATM: aztreonam (30 μg), CAZ: ceftazidime (30 μg), CRO: ceftriaxone (30μg), FEP: cefepime (30 μg), IMP: imipenem (10 μg), meropenem (10μg) as the MEM. CLSI breakpoint values were utilized to guide the interpretation.

DNA Extraction

DNA was extracted employing the boiling technique outlined by Yamamoto et al. (1995). A single colony was chosen using an inoculation loop from the MacConkey agar plate, transferred to a 1.5 mL autoclaved micro tube, mixed with 200 μL of distilled sterile water, placed on a foam plate, and boiled or 20 minutes (allowing the bacteria to release DNA). Once boiling was complete, the top and bottom were thoroughly combined, and the mixture was immediately placed into the refrigerator at -20 °C, and then kept frozen for ten to twelve minutes. This method was repeated three times, followed by centrifugation of the microtube at 10,000 rpm for 10 min, after which the supernatant was aspirated and kept as a backup at -20 °C to be utilized as the amplification template.

Detection of NDM gene by PCR and electrophoresis

PCR (Polymerase chain reaction) reactions with specific primers supplied by (Metabion International AG Company, Germany) were carried out to identify the bla NDM gene. The primers for NDM are as follows: Forward Primer (F): 5'-GGTTTGGCGATCTGGTTTTC-3' and Reverse Primer (R): 5'CGGAATGGCTCATCACGATC-3' (Poirel et al., 2011). The PCR was conducted in 20µl reaction volumes, consisting of 1µl each for the Forward and Reverse Primers, 2 µl of DNA template, 6 µl of nuclease-free water, and 10 µl of Taq Red Master Mix (Ampliqon, Denmark). DNA amplification was performed using a Thermal Cycler (DLAB, China) under the following conditions: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 20s, annealing at 58 °C for 30 s, 35 cycles, and extension at 72° C for 20s. For the detection of PCR products, mixing the products with 10 µl of loading dye, and 1.5% agarose gel electrophoresis (Invitrogen, USA) was employed to analyze the results for 45 minutes using 1X TBE running buffer. A 100 bp DNA ladder (SMOBIO Technology, Inc., Taiwan) was included in each run, and a UV transilluminator was used to view the DNA bands (Akhtarian, Iran) and then photographed.

Essential Oil extraction of Thymus vulgaris

Using a Clevenger-type apparatus, one hundred grams of *Thymus vulgaris* aerial parts were hydro-distilled for three hours following the European Pharmacopoeia procedure (2006). The essential oil was then stored at 4 °C in the dark in sealed vials. The major ingredients in the essential oil were thymol (38.23–63.01%), o-cymene (5.56–15.47%), γ-terpinene (4.43–7.17%), borneol (1.72–6.65%), 4-terpineol (1.24–5.16%), and 1, 8-cineole (0.09–1.54%). Prior to the analytical phase, the extracted oil was stored at a low temperature in a sealed vial after being deoxygenated using nitrogen gas (Nezhadali et al., 2014).

Antibacterial effect of Essential Oil extraction on clinically isolated

Agar well diffusion method

Thymus vulgaris essential (TVE) oil's antibacterial activities were determined by the standard disk diffusion susceptibility test on solid media (Mueller-Hinton agar) (Fani et al., 2017). The antimicrobial activity of TVE oil was tested in the Microbiology lab, Department of Microbiology and Microbial Biotechnology, in September 2023 at the Faculty of Life Science and Technology, Shahid Beheshti University, against ten distinct clinically isolated bacteria of *Klebsiella pneumoniae*. All isolates were obtained from Nasiriyah City, Iraq. One hundred microliters of each bacterium were suspended and adjusted to a density equivalent to 0.5 McFarland density standard. Using sterilized cotton swabs, the suspensions were distributed over the Mueller-Hinton agar plates, After that a well was created in the middle of the culture medium, and 50 microliters of the oil extract were applied to a well in the center of the plates. The plates were then incubated at 37°C for 24 hours for bacterial isolates, and the diameter of the inhibition zone (mm) was measured (Aziz et al., 2022).

Statistical analysis

The Statistical Analysis System data results from this study have been analyzed using GraphPad Prism 9 program and Microsoft Excel 2016 for each biological replicate. The level of probability at $P \leq 0.05$, which used to identify a significant difference.

Results

Prevalence of Klebsiella pneumoniae on different clinical sources.

One hundred twenty-seven clinical specimens believed to be *K. pneumoniae*, including sputum, blood, urine, burn, wound, and stool swabs, were obtained from both genders and different ages. They have been collected from various hospitals in the Thi-Qar Governorate, Iraq. *K. pneumoniae* was identified in 50 cases. The number and percentage of isolates were as follows. 17 (34%) isolates from urine, 9 (18%) blood, 12 (24%) sputum, 4 (8%) burn patients, 2 (4%) fluid, 3 (6%) from wounds infection, and 3 (6%) from stool samples. Furthermore, females were more affected, with 32 (64%) having *K. pneumoniae*, compared to males, with 18 (36%) (Table 1). Biochemical tests and the VITEK® 2 Compact system were used for the identification of the bacterial isolate.

Antibiotic susceptibility test

Antibiotic susceptibility testing (AST) results showed that Ampicillin had the highest resistance rate (100%), followed by Piperacillin (96%), Cefotaxime (84%), Ceftazidime (80%), Cefepime (76%), and Aztreonam (74%). The rates of resistance to Imipenem and Meropenem among the isolates were 66% and 68%, respectively (Figure 1), which displays the percentages of antibiotic resistance in a bar graph illustrating resistance percentages to eight different drugs and the GraphPad Prism v9 was used for data analysis.

DNA extraction and identification of bla NDM gene by PCR

The DNA extracted from 50 isolates of *Klebsiella pneumoniae* was obtained using the boiling method. The purity and concentration of DNA specimens ranged from (1.7-2) to (40-150) ng/µl, respectively. The results indicated that bla NDM was detected in isolates (32%, 16/50). All isolates in this study exhibited a PCR product with 621 bp using the *K.*

Table 1. Percentage of *Klebsiella pneumoniae* on different clinical sources

Type of specimen	Number of isolates	*K. pneumoniae* isolates (%)
Urine	17	34
Blood	9	18
Sputum	12	24
Burn Swab	4	8
Wound Swab	3	6
Stool	3	6
Fluid	2	4
Total	50	100

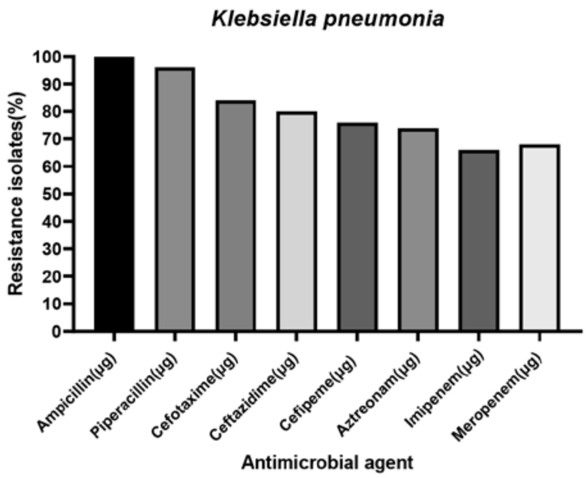

Fig. 1. Antimicrobial resistance frequencies in K. *pneumoniaee* isolates

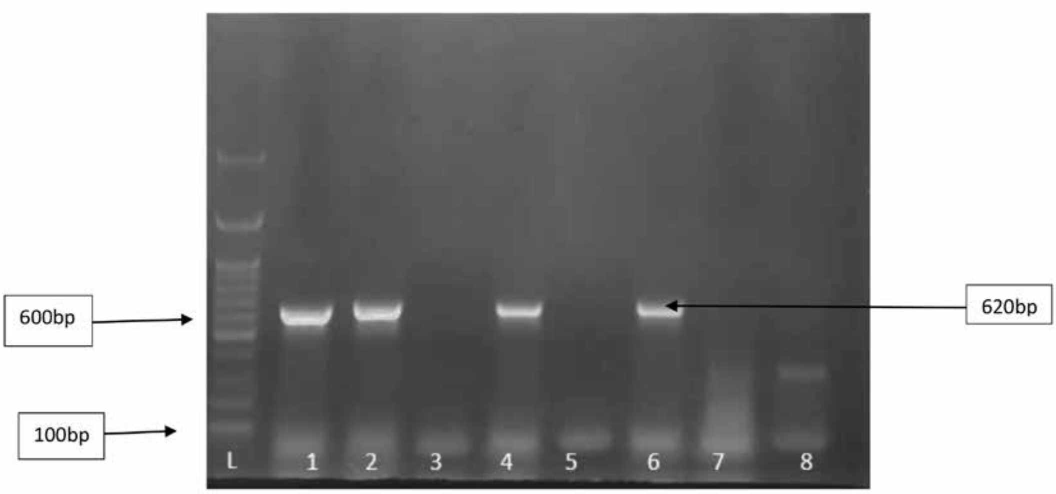

Fig. 2. Agarose gel electrophoresis of *bla* NDM gene(621pb) using specific primers of *K. pneumoniae* , L: DNA Ladder100- 1500 bp. 1, 2, 4, and 6 bands of bla NDM, and 3, 5, 7, and 8 negative isolates, agarose: 1.5%, 45 min at 100v

Table 2. Antibiotics resistant profile of multidrug-resistant *K. Pneumoniae* isolates

Antimicrobials	N6	N9	N10	N24	N26	N36	N38	N40	N44	N104
TVO	18mm R	35mm S	32mm S	48mm S	50mmS	30mm S	23mm(S) 32mm(S)	21mm(R) 40mm(S)	40mm S	16mm(R) 40mm(S)
Ampicillin	R	R	R	R	R	R	R	R	R	R
Piperacillin	R	R	R	R	R	R	R	R	R	R
Cefotaxime	R	R	R	R	S	R	S	R	R	R
Ceftazidime	R	R	R	R	S	R	R	R	R	S
Cefepime	R	R	R	R	S	R	S	R	R	S
Aztreonam	R	R	R	R	R	S	R	R	S	S
Imipenem	R	R	S	R	R	S	S	R	R	S
Meropenem	R	R	R	R	R	S	R	S	S	S

Abbreviations: TVO, *Thymus vulgaris* oil; R, resistance; S, sensitive; N, isolate number

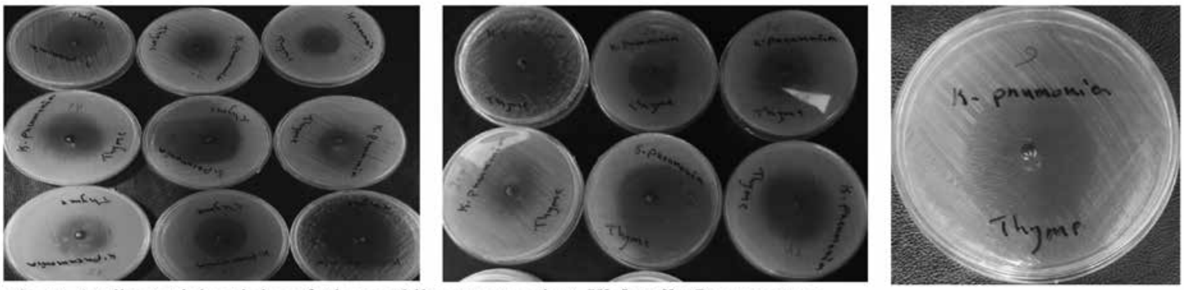

Fig. 3. Antibacterial activity of Thyme Oil extract against *Klebsiella Pneumoniae*

pneumoniae specific primer (bla NDM) designed for *K. pneumoniae* (Figure 2).

Antibacterial effect of Essential Oil extraction on isolated K. pneumoniae

The results show that the oil extract killed gram-negative bacteria (*K. pneumoniae*) used in this study. *K. pneumoniae* was the most sensitive to the essential oil extract, inhibiting growth with zones of inhibition ranging from 16 to 50 mm, as indicated by Table 2 and Figure (3), respectively.

Discussion

Multidrug-resistant (MDR) *K. pneumoniae* is a pathogen that can cause septicemia, lung infections, liver abscesses, and urinary tract infections. It is considered an opportunistic pathogen in both hospital and community-acquired settings (Muhsin et al., 2022). They account for 8-10% of all nosocomial bacteria in the United States and Europe (Podschun and Ullmann, 1998). A significant public health issue is the MDR strain of the prevalent nosocomial pathogen *K. pneumoniae*, which frequently causes diseases that are challenging to treat worldwide (Shahid et al., 2022).

Various diseases are treated with a wide variety of therapeutic plants and their extracts. *Thymus vulgaris* is one of the plants that is studied the most. Various chemical types of this plant's essential oils have already undergone testing and have demonstrated their antifungal and antibacterial properties, albeit with varying degrees of efficacy against certain microorganisms (Horváth et al., 2021). Essential oils are comparatively more efficient against Gram-positive bacteria than gram-negative bacteria, according to the majority of studies on their effects against foodborne pathogens and food spoilage organisms (Diniz et al., 2023). In this study, clinically isolated bacteria were employed to assess the antibacterial *Thymus vulgaris* leaf oil's effectiveness extracts against *K. pneumoniae*. The results presented in this study showed strong inhibitory activity of *Thymus vulgaris* oil extract on clinically isolated bacterial species, including *K. pneumoniae*, measured by agar well diffusion. The most sensitive strains to *Thymus vulgaris* essential oil produced the widest growth inhibition zone on the disk 48, and 50 mm (Table 2 and Figure 3). These results are not by Dosary (2018) in Saudi Arabia, where Thymus extracts did not affect any of the clinical isolates of *K. pneumoniae*. The current study's findings were in agreement with a study done by Aziz et al. (2022) using agar well diffusion, which found that the inhibition zone of Thymus vulgaris leaves oil extracts for *K. pneumoniae* was (28) mm. In a different study done by Asbaghian et al. (2011), they demonstrated the minimum inhibitory concentration (MIC) of the essential oil of *Thymus vulgaris* was determined to assess its antibacterial activity against strains of *Escherichia* coli and *K. pneumoniae*. This was achieved using the broth dilution method. Their findings showed that high concentration of active compounds, including thymol, carvacrol, 1,8-cineol, β-p ethyl alcohol, and γ terpinene, allowed the essential oil of *Thymus vulgaris* to effectively inhibit both bacteria. In another study conducted by Aziz et al. (2022), thyme oil had no antibacterial effect on *Pseudomonas aeruginosa*, and this was in contrast with our study.

As was previously mentioned, *K. pneumoniae* has an intrinsic defense mechanism called a capsule, which prevents Eos from penetrating the delicate inner membrane. Furthermore, in this direction, complex enzyme systems ought to contribute. The antibacterial activity of these compounds may vary depending on where one or more functional groups are located. Although thymol and carvacrol share a structural similarity, the two compounds' hydroxyl groups are located differently (Fournomiti et al., 2015). Because of their lipophilic characteristics, which allow them to readily penetrate bacterial cells and release components of cell membranes into the surrounding environment, essential oils have an antibacterial impact. Furthermore, research has shown that phenolic substances like thymol can damage Gram-negative bacteria's outer membrane, making the cytoplasmic membrane more permeable to ATP, and preventing biofilm formation. Thymus vulgaris essential (TVE) oil had a less potent effect. As thymol has been shown in multiple studies to be able to limit bacterial growth, this chemical may be primarily responsible for TVE's antibacterial actions (Dehghani et al., 2019).

The findings of the present investigation indicated that *K. pneumoniae* was isolated more frequently from urine. The high frequency of these bacteria in urinary tract infections in high numbers may be because these bacteria are considered part of the resident flora in the lower gastrointestinal tract and have virulence factors that contribute to their pathogenicity (Ali et al., 2017). As shown in Figure 1, This outcome agrees with a study done in the Iraqi city of Sulaymaniyah, which reported a higher percentage of urine samples (23, 42.6%). This indicates that urinary tract infections are the most prevalent infection in medical facilities, and *K. pneumoniae* is the second most frequently isolated bacteria after *E. coli* in this condition (Mohammed et al., 2022). In a previous local study conducted by Aziz Al Marjani (2022), it was found that among 50 *K. pneumoniae* isolates, 20 urine specimens tested positive for *K. pneumoniae.*

K. pneumoniae isolates exhibited a high resistance rate to most antibiotics. The resistance rate towards ampicillin recorded the highest percentage (100%), and piperacillin was at (96%). This result aligns with a local study by Ibrahim et al. (2022), who observed a 100% resistance to ampicillin. These elevated percentages are consistent with the findings of Conservation et al. (2023), which demonstrated that all isolates of *K. pneumoniae* were resistant to ampicillin in Hilla. Abbas et al. (2023) discovered a higher resistance rate for carbenicillin (99%), ampicillin (94.5%), and piperacillin (82.4%). The present study revealed a high rate of antibiotic β-lactam resistance, for example, Cefotaxime (84%), ceftazidime (80%), and additionally Cefepime (76%). This may result from people using these antibiotics frequently without proper medical care. This result is consistent with a local study by Jwair et al. (2023), which found a high rate of resistance to β-lactam antibiotics, including 83.0% for ceftriaxone, 84.0% for cefepime, and 85.0% for cefazolin.

Conclusion

According to this study, testing the combined effects of essential oils (*Thymus vulgaris*) had broad bactericidal activities, of which they demonstrated a stronger antibacterial effect against common ESBL-producing *K. Pneumoniae*. *Thyme vulgaris* essential oil exhibited strong antibacterial action against *K. pneumoniae*, giving it a promising natural agent for combating microbial infections. However, the highest antimicrobial effect observed was against *K. Pneumoniae*.

In addition, this finding could be important for further studies to identify, purify, and determine the precise role of the bioactive molecules responsible for this effect and discover potential uses for both the food industry and pharmaceutical applications.

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References

Ali FA and Ismael RM. (2017). Dissemination of *Klebsiella pneumoniae* and *Klebsiella oxytoca* Harboring bla TEM genes isolated from different clinical samples in Erbil City. Diyala Journal of Medicine. 12 (2): 40-51.

Asbaghian S, Shafaghat A, Zarea K, Kasimov F, Salimi F. (2011). Comparison of volatile constituents, and antioxidant and antibacterial activities of the essential oils of *Thymus caucasicus*, *T. kotschyanus* and *T. vulgaris*. Natural Product Communications. 6 (1). Doi: https://doi.org/10.1177/1934578X1100600133.

Aziz PY, Azeez SH, Hama NH, Taha Y. (2022). Antibacterial activity evaluations of Thymus vulgaris essential oil extract against clinically isolated gram-positive and gram-negative pathogens. 14 (2): 164–173.

Aziz SN and Al Marjani MF. (2022). Investigation of bacterial persistence and filaments formation in clinical *K. pneumoniae*. ARO-The Scientific Journal of Koya University. 10 (2): 82-86. Doi: https://doi.org/10.14500/aro.10895.

Chang D, Sharma L, Dela Cruz CS, Zhang D. (2021). Clinical epidemiology, risk factors, and control strategies of *Klebsiella pneumoniae* infection. Frontiers in Microbiology. 12: 750662. Doi: https://doi.org/10.3389/

fmicb.2021.750662.

Chen J, Li J, Huang F, Fang J, Cao Y, Zhang K, Zhou H, Cai J, Cui W, Chen C, Zhang G. (2023). Clinical characteristics, risk factors, and outcomes of *Klebsiella pneumoniae* developing secondary *Klebsiella pneumoniae* bloodstream infection. BMC Pulmonary Medicine. 23 (1): 1–11. https://doi.org/10.1186/s12890-023-02394-8

Christaki E, Marcou M, Tofarides A. (2020). Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. Journal of Molecular Evolution. 88 (1): 26-40. Doi: https://doi.org/10.1007/s00239-019-09914-3

Conservation ER, Abbas FM, Jarallah EM. (2023). First identification of NDM-1 Metallo ß – Lactamase among clinical isolates of *Klebsiella pneumoniae* isolates in Hilla hospitals. Iraq. 11 (2), 130-138.

Dalmolin TV, Bianchini, BV, Rossi GG, Ramos AC, Gales AC, Trindade PA, de Campos MMA. (2017). Detection and analysis of different interactions between resistance mechanisms and carbapenems in clinical isolates of *Klebsiella pneumoniae*. Brazilian Journal of Microbiology. 48 (3): 493-498. https://doi.org/10.1016/j.bjm.2017.01.003.

Dehghani N, Afsharmanesh M, Salarmoini M, Ebrahimnejad H. (2019). In vitro and in vivo evaluation of thyme (*Thymus vulgaris*) essential oil as an alternative for antibiotic in quail diet. Journal of Animal Science. 97 (7): 2901-2913. https://doi.org/10.1093/jas/skz179.

Diniz AF, Santos B, Santos VRL, Mariz WS, Cruz PSC, Silva RL, Paula AFR, Santos JRDA. (2023). Antibacterial activity of *Thymus vulgaris* (thyme) essential oil against strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus* isolated from the meat product. Brazilian Journal of Biology. 83: 1-9. Doi: 10.1590/1519-6984.275306.

Dosary SKAl. (2018). Antibacterial effect of Thymus sp . and Boswellia sp. extracts on *Streptococcus pneumoniae* and *Klebsiella pneumoniae* isolates. 17 (5): 133-138. https://doi.org/10.5897/AJB2017.16051

Fani M and Kohanteb J. (2017). In vitro antimicrobial activity of *Thymus vulgaris* essential oil against major oral pathogens. Journal of Evidence-Based Complementary and Alternative Medicine. 22 (4): 660–666. https://doi.org/10.1177/2156587217700772

Firoozeh F, Mahluji Z, Shams E, Khorshidi A, Zibaei M. (2017). New Delhi metallo-ß-lactamase-1-producing *klebsiella pneumoniae* isolates in hospitalized patients in Kashan, Iran. Iranian Journal of Microbiology. 9 (5): 283-287. PMCID: PMC5748447.

Fournomiti M, Kimbaris A, Mantzourani I, Plessas S, Theodoridou I, Papaemmanouil V, Kapsiotis I, Panopoulou M, Stavropoulou E, Bezirtzoglou EE, Alexopoulos A. (2015). Antimicrobial activity of essential oils of cultivated oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) against clinical isolates of *Escherichia coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*. Microbial Ecology in Health and Disease. 1: 1-7. Doi: 10.3402/mehd.v26.23289 .

Ghanbarinasab F, Haeili M, Ghanati SN, Moghimi M. (2023). High prevalence of OXA-48-like and NDM carbapenemases among carbapenem resistant *Klebsiella pneumoniae* of clinical origin from Iran. Iranian Journal of Microbiology. 15 (5): 609-615. https://doi.org/10.18502/ijm.v15i5.13866.

Han R, Shi Q, Wu S, Yin D, Peng M, Dong D, Zheng Y, Guo Y, Zhang R, Hu F. (2020). Dissemination of carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) among carbapenem-resistant enterobacteriaceae isolated from adult and children patients in China. Frontiers in Cellular and Infection Microbiology. 10: 314. Doi: https://doi.org/10.3389/fcimb.2020.00314.

Horváth G, Horváth A, Reichert G, Böszörményi A, Sipos K, Pandur E. (2021). Three chemotypes of thyme (*Thymus vulgaris* L.) essential oil and their main compounds affect differently the IL-6 and TNFα cytokine secretions of BV-2 microglia by modulating the NF-κB and C/EBPβ signalling pathways. BMC Complementary Medicine and Therapies. 21 (1): 1-14. Doi: https://doi.org/10.1186/s12906-021-03319-w.

Ibrahim DH, Abdullah BH, Abdulqadir IM. (2022). Detection of multi-drug resistant *Klebsiella Pneumoniae* from sputum samples among ICU patients utilizing pcr and Vitek2 System. The Journal of University of Duhok. 25 (2): 473-481. Doi: https://doi.org/10.26682/sjuod.2022.25.2.43

Jwair NA, Al-Ouqaili, MTS, Al-Marzooq F. (2023). Inverse association between theeExistence of CRISPR/Cas Systems with antibiotic resistance, extended spectrum β-Lactamase and carbapenemase production in multidrug, extensive drug and pandrug-resistant *Klebsiella pneumoniae*. Antibiotics. 12 (6). https://doi.org/10.3390/antibiotics12060980.

Kwiatkowski P, Sienkiewicz M, Pruss A, Łopusiewicz Ł, Arszyńska N, Wojciechowska-Koszko I, Kilanowicz A, Kot B, Dołęgowska B. (2022). Antibacterial and anti-biofilm activities of essential Oil compounds against New DelhiMetallo-β-Lactamase-1-producing uropathogenic *Klebsiella pneumoniae* strains. Antibiotics. 11 (2). Doi: https://doi.org/10.3390/antibiotics11020147

Li Y, Kumar S, Zhang L, Wu H. (2022). *Klebsiella pneumoniae* and Its antibiotic resistance: A bibliometric analysis. BioMed Research International. Doi: https://doi.org/10.1155/2022/1668789.

Liang Z, Wang Y, Lai Y, Zhang J, Yin L, Yu X, Zhou Y, Li X, Song Y. (2022). Host defense against the infection of *Klebsiella pneumoniae*: New strategy to kill the bacterium in the era of antibiotics? Frontiers in Cellular and Infection Microbiology. 12: 1050396. Doi: https://doi.org/10.3389/fcimb.2022.1050396

Mohammed AB and Anwar KA. (2022). Phenotypic and genotypic detection of extended spectrum beta lactamase enzyme in *Klebsiella pneumoniae*. PLOS ONE. 17 (9): 1–14. Doi: https://doi.org/10.1371/journal.pone.0267221

Muhsin EA, Said A, Al-Jubori SS. (2022). Correlation of type 1 and Type 3 fimbrial genes with the type of specimen and the antibiotic resistance profile of clinically isolated *Klebsiella pneumoniae* in Baghdad. Al-Mustansiriyah Journal of Science. 33 (3): 1-11. Doi: https://doi.org/10.23851/mjs.v33i3.1129

Nezhadali A, Nabavi M, Rajabian M, Akbarpour M, Pourali P, Amini F. (2014). Chemical variation of leaf essential oil at different stages of plant growth and in vitro antibacterial activity of *Thymus vulgaris* Lamiaceae, from Iran. Beni-Suef University Journal of Basic and Applied Sciences. 3 (2): 87-92. Doi: https://doi.org/10.1016/j.bjbas.2014.05.001.

Osama D, El-Mahallawy H, Mansour MT, Hashem A, Attia AS. (2021). Molecular characterization of carbapenemase producing *klebsiella pneumoniae* isolated from Egyptian pediatric cancer patients including a strain with a rare gene-combination of βlactamases. Infection and Drug Resistance. 14: 335–348. Doi: https://doi.org/10.2147/IDR.S284455

Podschun R and Ullmann U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clinical Microbiology Reviews. 11 (4): 589-603. Doi: https://doi.org/10.1128/cmr.11.4.589

Poirel L, Walsh TR, Cuvillier V, Nordmann P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. Diagnostic Microbiology and Infectious Disease. 70 (1): 119-123. Doi: https://doi.org/10.1016/j.diagmicrobio.2010.12.002

Shahid M, Ahmad N, Saeed NK, Shadab M, Joji RM, Al-Mahmeed A, Bindayna KM, Tabbara KS, Dar FK. (2022). Clinical carbapenem-resistant *Klebsiella pneumoniae* isolates simultaneously harboring bla NDM-1, bla OXA types and qnrS genes from the Kingdom of Bahrain: resistance profile and genetic environment. Frontiers in Cellular and Infection Microbiology. 12: 1033305. Doi: https://doi.org/10.3389/fcimb.2022.1033305.

Lobo AS and Moosabba M. (2020). Antibiogram and hypermucoviscosity pattern among *Klebsiella pneumoniae* isolate from respiratory samples: A tertiary care hospital study in South India. IP International Journal of Comprehensive and Advanced Pharmacology. 4 (4): 134-138. https://doi.org/10.18231/j.ijcaap.2019.028.

Yamamoto S, Terai A, Yuri K, Kurazono H, Takeda Y, Yoshida O. (1995). Detection of neurovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immunology and Medical Microbiology, 12 (2): 85-90. Doi: https://doi.org/10.1016/0928-8244(95)00053-A