

ORIGINAL ARTICLE

Comparative Study of *Malva parviflora* and Antibiotics on Biofilm Formation of *klebsiella pneumoniae* Isolates

Rana Mohammed Salman^{1*}

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Competing Interests:

The authors declare that this manuscript was approved by all authors in its form and that no competing interest exists.

Affiliation and Correspondence:

University of Diyala, Diyala. Iraq
Email:

*Corresponding Author Email:

ranamohammed@uodiyala.edu.iq

ABSTRACT:

Background: The spread of *Klebsiella pneumoniae* Multidrug-Resistant (MDR) poses a major health threat negatively impacting both young and elderly age groups.

Objective: The study aimed to assess the prevalence of *K. pneumoniae* infections among hospitalized patients, and to determine the sensitivity of the bacteria to common antibiotics. Estimate the MIC and Sub MIC for some antibiotics used in treatment of *K. pneumoniae* infections. Also, study the effect of aqueous and alcoholic extracts of *Malva parviflora* on biofilm formation.

Methods: The clinical samples under study taken from 90 patients, who visited or were admitted to Baqubah Teaching Hospital in Diyala.

Results: The results showed that biofilm formation was obtained in 13 *K. pneumoniae* isolates, and antibiotic susceptibility testing against isolates, high resistance to most of the 14 antibiotics used in this study. It is noteworthy that the isolates recorded 100% resistance to Ampicillin and Cefotaxime. Sub MIC and MIC of Ciprofloxacin and Trimethoprim-Sulfamethoxazole which ranged from 4 to 1024 µg. While the results showed that the aqueous and alcoholic extracts various in effectiveness against the bacterial isolates, the alcoholic extracts showed higher effectiveness, with the diameter of the inhibition zone reaching 30mm against the isolates. This effect is attributed to the high efficiency of alcoholic extracts in extracting chemical compounds with antibacterial activity.

Conclusion: In conclusion, the medicinal efficacy of *M. parviflora* extracts can be attributed to the presence of active compounds. Our results showed that the extracts have a biological activity against the formation of biofilms of the clinical isolates studied, which indicates the possibility of using these extracts in the treatment of infectious diseases.

KEYWORDS: *Klebsiella pneumoniae*, Biofilm formation, Antibiotics Resistant, *Malva parviflora*

INTRODUCTION:

Klebsiella pneumoniae is a serious pathogen that causes hospital-acquired and community-acquired urinary tract infections. And it causes serious infections in immune-compromised individuals, such as new-borns, the elderly, and hospitalized patients. Currently, highly virulent strains have spread to healthy, Non-Immunocompromised young adults (Seifi et al., 2016). These bacteria are becoming increasingly resistant to antibiotics, limiting available treatment options. Initially, *K. pneumoniae* is one of the opportunistic pathogens that responsible for many hospital-acquired infections, particularly those involving the renal and respiratory systems. This bacterium can also cause soft tissue and skin infections, wound infections, bloodstream infections, and meningitis (Sleiman et al., 2021). The patho-genicity of *K. pneumoniae* depends on specific genes that produce virulence factors, which disrupt the mammalian immune system and cause various diseases (Ahmadi et al., 2022). *K. pneumoniae* is the major agent of multidrug-resistant (MDR) infections worldwide. The main challenges are the mechanisms of multiple antibiotic resistance. It has resistance to quinolones, aminoglyco-sides, carbapenems, and cephalosporins, as well as the need to understand the mechanisms of resistance evolution to improve and prevent treatment (Qian et al., 2023; Si et al., 2023). It has several virulence factors that it shares with other pathogens, including capsule antigens, adhesion factors, enterotoxin producing lipopolysaccharides and Biofilm formation, as well as these factors enable it to evade the host's innate immune mechanisms (Riwu et al., 2022).

Biofilms are interconnected microbial communities within an external matrix where microorganisms interact and influence each other, creating a complex and stable microbial environment. It is composed of polysaccharides, proteins, lipopeptides, and DNA. They may be monolayered or multilayered. Biofilm formation is driven by quorum-sensing mechanisms and gene regulation. Biofilms play an essential and main role in promoting antibiotic resistance and increasing the virulence of bacteria, it they provide a protected environment, reducing the effectiveness of antibiotics and making infections more difficult to treat, and an additional layer of protection against the host immune response and antibiotic therapy. This complicates the treatment of *K. pneumoniae* infections, especially in the case of multidrug-resistant isolates (Jasim et al., 2020). The search for effective, safe, and reliable sources of drugs is a constant drive for the development of new forms of pharmaceutical preparations for the treatment of various diseases. Since the mid-20th century. Plant-derived phytochemicals have been used to develop widely used antimicrobial drugs after careful

testing in humans. Clinical microbiologists are interested in plant-derived phytochemicals to develop new and effective drugs (Caesar and Cech, 2019).

Malva parviflora L. is a perennial herbaceous plant belonging to the Malvaceae family. It is widely distributed in tropical, subtropical, and temperate regions of Asia, Africa, and Europe. *M. parviflora* is commonly known as "cheese weed". Its broad leaves have 5-7 lobes and are 8-10 cm in diameter. Additionally, the plant produces small white or pink flowers with petals measuring 4-6 mm. *M. parviflora* contains a variety of chemical compounds, including, Glycosides, alkaloids, steroids, flavonoids, terpenes, saponins, and tannins in methanol extract (Estabraq et al., 2022). *M. parviflora* is used in traditional medicine and food, and has important uses in health. The main polyphenolic components in the plant leaf extracts were identified using high-performance liquid chromatography (HPLC), which include: *p*-coumaric acid, naringin, luteolin, cinnamic acid and apigenin-7-glucoside (Heydarirad et al., 2017). Although numerous studies have demonstrated the general antimicrobial activity of plant extracts, research focusing on using *M. parviflora* extracts to inhibit biofilm formation in *K. pneumoniae* isolates is still very limited. Previous studies have indicated the antimicrobial activity of alcoholic extracts of *M. parviflora* against respiratory and skin pathogens, with the highest inhibition zone being 24 mm against *Haemophilus influenzae*. At the same time, it was lower against *Staphylococcus aureus*, with a 15 mm (Madisha, 2024). In a study conducted by Ododo et al., (2016), the antimicrobial activity of the chloroform extract of *M. parviflora* against *Escherichia coli* and *Staph aureus* was documented at (15±0.41mm) and MIC value of 20 mg/ml, while the activity of the ethanolic extract was documented against only *Staph aureus* (18±3.20mm), with MIC value of 15 mg/ml. However, its effectiveness against resistance mechanisms, including biofilm formation, has not yet been adequately explored and studied. Therefore, this study focused on a systematic investigation to clarify and demonstrate the inhibitory effect of the plant extracts on biofilm formation.

We hypothesize that *M. parviflora* extracts will demonstrate significant antimicrobial activity against biofilm formation in *K. pneumoniae*. The current study aimed to: Evaluate the prevalence of drug-resistant *K. pneumoniae* in Baqubah hospital. Which determines the sensitivity and resistance of bacteria to common antibiotics and estimate Minimum Inhibitory Concentration (MIC) and Sub-Minimum Inhibitory Concentration (Sub- MIC) of some antibiotics to inhibit Biofilm formation in bacteria. Additionally, It's aimed to study the antimicrobial activity in inhibiting biofilm formation of *K. pneumoniae* isolates by using the alcoholic and aqueous extracts of *M. parviflora*, which will show

inhibitory activity against the growth and biofilm formation of the isolates. Thus supporting their use and providing a rich source of effective antimicrobial compounds.

MATERIALS and METHODS

Collection of bacterial samples

A total of 90 clinical samples were collected from Baqubah Teaching Hospital in Diyala Province. The process of sampling collection lasted for 3 months (10/11/2023 to 31/1/2024). filling out a questionnaire for all patients to collect medical information: age, gender, health problems, medication intake and history of health deterioration. Clinical samples collected and these transfer to the Laboratory for analysis, Samples are cultured on prepared media. for Diagnosis of the samples .

Isolation and Diagnosis of K. pneumonia

After collecting the samples and transferring them to the laboratory, they were cultured on fresh MacConkey agar at 37°C for 24 hours. Based on the initial diagnostic tests that depended on examining of morphological characteristics of bacterial growth, Than were cultured on Eosin Methylene Blue as a selective media for the K. pneumoniae and confirmed by biochemical tests, Such as oxidase test, catalase test, IMViC tests, urea hydrolysis, haemolysis and motility test (Macfaddin, 2000). All the results of these tests were confirmed by Vitek-2 compact system.

Antibiotic Susceptibility Test for K. pneumoniae

By using the Kirby–Bauer method to determine the sensitivity of K. pneumoniae to antibiotics (Höring et al.,2019). The test involved analysing the bacteria's sensitivity to 14 common antibiotics and comparing with CLSI, (2020). Were used modern generation for it used to treat of diseases causes by K. pneumoniae, namely Ampicillin (AM - 25 µg), Ceftriaxone (CTR-30 µg), Amikacin (AK -10 µg), Cefotaxime (CTX -30 µg), Cefoxitin (CX -30 µg), Ceftazidime (CAZ-30µg), Ciprofloxacin (CIP-10µg), Tobramycin (TOB-10µg), Meropenem (MRP-10µg), Amoxicillin- clavulanic acid (AMC-30µg), Doxycycline(DO-10µg), Azithromycin (AZM 15 µg), Imipenem (IMP-10µg), and Trimethoprim- sulfa methoxazole (COT-25 µg).

Bacteria have numerous mechanisms of antibiotic resistance. Genetic mutations within the cell, or those acquired by the cell, contribute to the development of resistance through spontaneous mutations. These genes are associated with transposons, integrons, and plasmids (Sinoov and Steberg,

2022). Bacteria may also possess beta-lactamase enzymes that break down the beta-lactam ring in the beta-lactam antibiotic group, disrupting the action of antibiotics such as penicillin and cephalosporins. This increases the rate of resistance of isolates to this class of antibiotics, which is common in *K. pneumoniae* (Bannin et al., 2023). Another resistance mechanism for isolates is the formation of a biofilm, which slows or prevents antibiotic penetration through the membrane, thus increasing the resistance rate (Jafari-Saks et al., 2020). Bacteria also have efflux pumps, which are proteins that expel substances and antibiotics out of the bacterial cell, thus enabling one of the mechanisms of antibiotic resistance (Boren et al., 2020) .

Biofilm formation

The microtiter plate method was used to study the biofilm formation of bacterial isolates. The method involved culturing bacteria for 24 hours at 37°C, followed by transferring colonies to brain and heart infusion broth (BHB) medium. The bacterial suspension was then spread onto a 96-well polystyrene plate and incubated for 24 hours. Followed by incubation, the biofilms were stained with crystal violet, and the absorbance was measured at 630 nm using an ELISA microplate reader. This method allows for the determination of the ability of bacteria to form biofilms (Jesus and Dedeles, 2020). Previous studies have shown that the ability to form biofilm varies among the isolates under study. This is due to the influence of various factors, including the nutrient level of the microbial community within the biofilm, The Concentration of dye, as a dye of concentration 5% gives a greater density than if it were 1% and the incubation conditions of the isolates, the membrane density increases when the period of incubation was long (Farooq et al., 2021). Additionally, differences in the materials composing the surfaces on which the biofilm forms can affect its effectiveness. In other words, a biofilm formed on a particular surface may be more effective than one formed on another (Slettengren et al., 2020) .

Determination of Minimum, Sub- Minimum Inhibitory Concentration

Concentration (MIC, Sub MIC) for antibiotics (Ciprofloxacin (CIP) and Trimethoprim-sulfamethoxazole (COT), were determined for toward *K.pneumoniae* isolates The values of them for all of the isolates were determined according to Andersson and Hughes, (2016).

Collection of plant samples

We collected a sample of *M. parviflora* in Baqubah city in January 2024. After collection, the leaves

were separated from the stems and washed with distilled water to ensure their cleanliness. The leaves were then dried in the shade for two weeks at room temperature, making sure not to expose them to direct sunlight. After drying, the leaves were manually crushed using a grinder or beater to prepare a fine, homogeneous powder. The powder was then stored in a tightly sealed container made of glass or plastic, in a dry and cool place at room temperature, making sure not to expose it to moisture or direct light .

Preparation of plant extracts

The plant extract was prepared using ethanol (70%) by soaking 50 g of plant powder in 500 ml of solvent for 24 h in a shaking incubator at 28°C. After filtration, the extract was concentrated using a vacuum rotary evaporator to obtain a thick liquid and then dried in an incubator at 37°C for 48 h. The dry extract was stored in a tightly sealed tube wrapped in aluminium foil in a refrigerator at 4°C until use. This method allows for the preparation of a concentrated, dry plant extract that can be used in subsequent studies. Based on what was stated by Dakhil et al., (2023). Ethanolic and Aqueous extracts of the *M. parviflora* L. were prepared. As is well known, the solvent used in the extraction process of active compounds plays a fundamental and important role in determining the type of active compounds extracted from plant tissues. Alcohols (ethanol and methanol) are medium-polar solvents and are highly effective in extracting medium-polar compounds (tannins, terpenes, flavonoids), which are often associated with antimicrobial activity. On other hand, Water is highly polar and targets highly polar compounds (Amino acids, polysaccharides), So the difference in biological activity between extracts reflects the difference in extraction efficiency and dissolution resulting from the dissolving action of the solvent (Faeji et al., 2024; Lezoul et al., 2020).

Determination of Antimicrobial Activity of M. parviflora extracts

According to Taherkhani et al., (2013), using the well diffusion agar method to determine the antimicrobial activity of *M. parviflora* extracts, bacterial suspensions of the isolates under study were prepared in tubes containing Heart Brain infusion broth medium. A portion of the bacterial colonies was transferred by sterile loop to these tubes. The turbidity of the bacterial suspension was compared to the McFarland standard (1.5×10^8). Using a sterile swab, the bacterial suspension was spread on Muller Hinton agar medium and then allowed to dry. A cork drill was used to make 4 holes in the culture medium with a diameter of 5 mm. 1 g of the dry prepared extract powder was transferred to tubes 2 ml of distilled water, and 2 ml of each tube of ethanol and methanol. These extracts were

sterilized with a Millipore filter paper with a diameter of 0.22 μ m (stock solution). A series of dilutions was made to obtain concentrations of (50, 100, 200) mg/ml. Using a pipette, a portion of about 4.1-4.9 ml of each concentration of extracts was transferred to each well. The fourth well was filled with distilled water as a negative control, compared with the other well after incubation for one day at 37°C .

Statistical analysis

The researcher used analysis and interpretation of the data by appropriate statistical means and with the help of the statistical program (SPSS) as follows:

- First: Variables were described in the form of numbers and percentages .
- Second: The t-test was used for numerical comparisons. A p-value <0.005 was significant. A one-way ANOVA test was used to compare the mean inhibition areas of groups (Levesque, 2007).

RESULTS

Isolation and Diagnosis of *K. pneumoniae*

The samples were collected from patients (90) cultured on Machonkey agar medium 72/90(80%) from samples gave a positive result for growth and negative growth results were 18/90 (20%), After that transferred to (EMB) medium to isolate the *K. pneumoniae*, 19 isolates (26.39%) were distinguished Figure 1. large sticky pink colonies. The distinctive feature of *K. pneumoniae* (Rameshkumar et al., 2015), were confirmed presence of bacteria in samples.

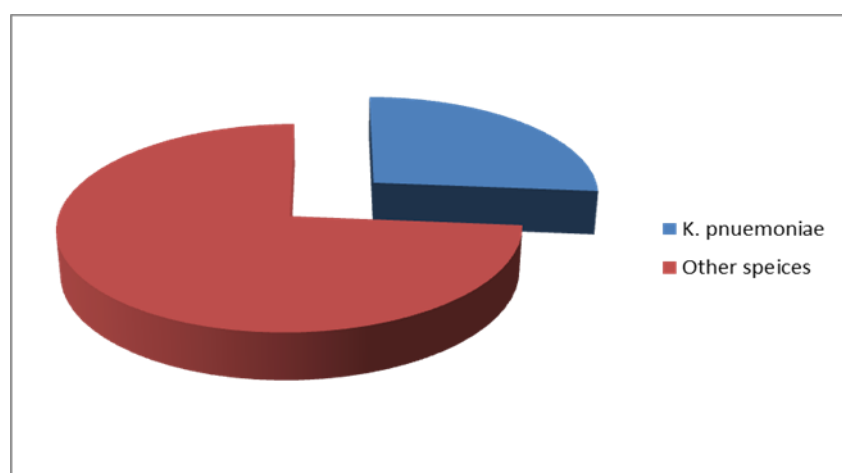


Figure 1 .The percentage of growth in *k. pneumoniae*

The microscopical examination of 19 samples confirmed that isolates appeared as Pink rod-shaped bacillus species arranged in pairs or single (Mahon and Lehman, 2022). Biochemical tests were also

used based on the mention in Riedel et al., (2019) Table 1 .

Table 1. Biochemical tests identification criteria of k. pneumoniae isolates

Biochemical Test	Oxidase	Catalase	Indole	Methyl Red	Voges-proskaur	Citrate	Urase	Motility
<i>K. pneumoniae</i>	-	+	-	-	+	+	+	-

The diagnosis was confirmed using the Vitek-2 Compact system. The present study focused on K. pneumoniae infections in age groups Children with an immature immune system and Elderly people (over 50 years of age), with a weakened immune system due to old age. The study was conducted in winter, when the incidence of infection was higher in these two age groups. The results of the current study showed a high percentage isolation of K.pneumoniae, which reached (26.39%), These results were very close to the study of Mohammad et al.,(2023), which reached.(%٢٤,١٦)

Detection of Biofilm Composition of Isolates

To test Biofilm formation of bacterial isolates using the Microtiter Plate Method, to study Biofilm formation on the surface of isolates, which is a colorimetric technique that using crystal violet-dye to stain it, And by use an absorbance microtiter plate reader (Jesus and Dedeles, 2020).19 isolates were evaluated to test the ability of Biofilm formation. 13/19 (68.42%) isolates were forming it, while 7/19 (31.58%) isolates were non-forming it. The isolates were showed biofilm forming properties and their absorbance value, which was 0.052. 9/13 (69.23%) of isolates were strong biofilm-forming, while 4/13 (30.77%) were moderate. These results were closer to the study results of Alobadi et al., (2022) 49% was strong, Table2

Antibiotic Sensitivity Test of K. pneumoniae

As shown in Figure 2. That 13 bacterial isolates Biofilm formation were tested against 14 antibiotic agents in current study, It showed strong resistance to antibiotics, especially Ampicillin 100%, This results were agree with Al-Zubadi and Al Taai, (2020) and Abdo, (2022) it was 100%. The resistance of isolates to Cefotaxime and Ceftizidim, Showed high resistance 100% and 92.3% of bacterial isolates in this study, Which agree with finding Mohammed et al., (2023), Who recorded 96.6% a resistance of isolates to both Cefotaxime and Ceftizidime, The most of isolates also showed high resistance rates of Ceftriaxone at 84.6%. the agreement of Muhsin et al., (2022), was 80%. In terms of Flouroquinolones, the isolates in the current study recorded a rate of resistance of 69.2% against Ciprofloxacin. In

comparison, Mohammed et al., (2023) reported 41.4%. .

Table 2. Biofilm formation for k. pneumonia

No. of the isolates	Absorbency at 630 nm	Biofilm formation compared to (OD=0.052) (2*ODc=0.117)
<i>K. pneumoniae</i> 1	0.129	Strong
<i>K. pneumoniae</i> 2	0.080	Moderate
<i>K. pneumoniae</i> 3	0.136	Strong
<i>K. pneumoniae</i> 4	0.129	Strong
<i>K. pneumoniae</i> 5	0.081	Moderate
<i>K. pneumoniae</i> 6	0.124	Strong
<i>K. pneumoniae</i> 7	0.079	Moderate
<i>K. pneumoniae</i> 8	0.126	Strong
<i>K. pneumoniae</i> 9	0.066	Moderate
<i>K. pneumoniae</i> 10	0.118	Strong
<i>K. pneumoniae</i> 11	0.086	Moderate
<i>K. pneumoniae</i> 12	0.122	Strong
<i>K. pneumoniae</i> 13	0.055	Moderate

a. OD" Mean optical density reader average of isolates b. ODc" Mean optical density reader average of control

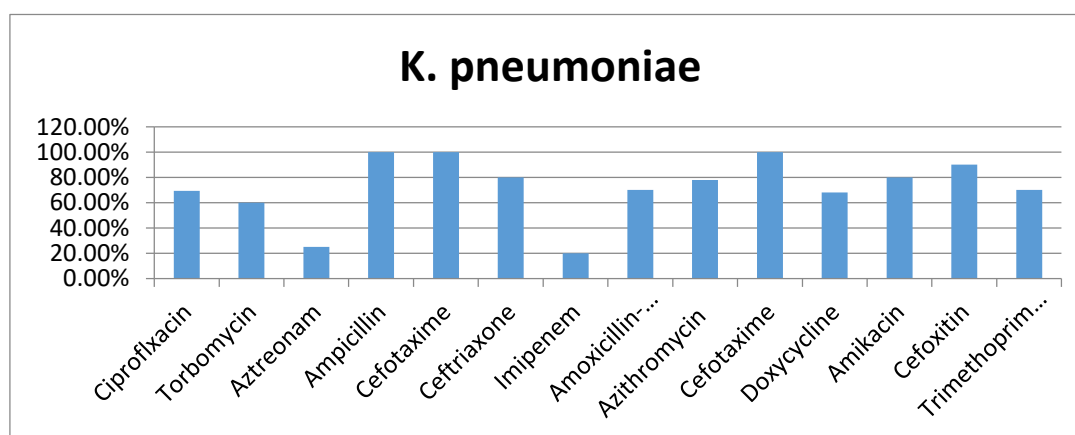


Figure 2. Antibiotic Resiatance of K. pneumoniae isolates(n=13).

Furthermore, isolates of K. pneumoniae showed moderate resistance to Azithromycin, 76.9%, according to study by Ahmed et al., (2020), at 65%. The reason for resistance most of isolates possessing erm genes that inhibit interaction Azithromycin with the ribosome, Thus Inhibition of protein synthesis (Liu et al., 2022). Resistance to Imipenem and Meropenem were 30.7% and 38.4% against isolates under study, this result was consistent with the results of another study conducted by Davoudababdi et al., (2023). The rate resistance was 28% to Meropenem and 42% to Imipenem. The results of the current study showed that 76.9% of the isolates were resistant to the Aminoglycoside class, including Amikacin.This resistance due to what was mentioned by Lia et al., (2022).

The study demonstrated that the highest rate of isolates resistance among isolates to Amoxicillin-

Clavulanic acid (AMC) was 69.2%. These results were consistent with those documented by Caneriras et al., (2019). Also, the results recorded high resistance to Ampicillin, which was 100% in this study. This results were agree with Al-Zubadi and Al Taai, (2020), and Abdo, (2022), which was 100% .

Minimum and Sub-Minimum Inhibitory Concentration of Antibiotics against Isolates

Table 3, presents the Sub-MIC and MIC analysis of highly antibiotic- resistant isolates of *K. pneumoniae* Biofilm forming (Strong and Moderate), on Ciprofloxacin and Trimethoprim-Sulfunmethoxazole, which is the lowest concentration at which bacteria can grow, while the values of MIC were determined by calculating the MIC for the growth of each isolate under study. By using Muller Hinton Broth with the Serial dilution method, We can measure absorbance and read it in a Microplate reader. After incubation for 24hours, MIC and sub-MIC were recorded for all isolates as shown in Table 3.

Table 3. Sub-MIC and MIC values ($\mu\text{g/ml}$) of antibiotics against *K. pneumonia*

No. of the isolates	Ciprofloxacin ($\mu\text{g/ml}$)		Trimethoprim-Sulfamethoxazole ($\mu\text{g/ml}$)	
	Sub MIC	MIC	Sub MIC	MIC
<i>K. pneumoniae</i> 1	32	64	512	1024
<i>K. pneumoniae</i> 2	4	8	512	1024
<i>K. pneumoniae</i> 3	32	64	512	1024
<i>K. pneumoniae</i> 4	16	32	32	64
<i>K. pneumoniae</i> 5	4	8	32	64
<i>K. pneumoniae</i> 6	32	64	512	1024
<i>K. pneumoniae</i> 7	32	64	256	512
<i>K. pneumoniae</i> 8	16	32	4	8
<i>K. pneumoniae</i> 9	32	64	512	1024
<i>K. pneumoniae</i> 10	32	64	256	512
<i>K. pneumoniae</i> 11	32	64	256	512
<i>K. pneumoniae</i> 12	32	64	32	64
<i>K. pneumoniae</i> 13	4	8	512	1024

Effect of some Antibacterial agents on Biofilm Forming Bacterial Isolates

It is known that the formation of bacterial isolates in Biofilm is more resistant to antibacterial agents. However it may be inhibited stimulation and formation when planktonic cells are exposed to sub-MIC of some antibacterial agents. Biofilm is a large health problem (Otani et al., 2018). In this study, the effect of some Antibacterial agents was studied (Ciprofloxacin and Trimethoprim-Sulfunmethonozole) On formation of Biofilm to each isolate (Strong and Moderate) under study, Sub MIC values were recorded. The results are illustrated in Table 4. Showed a decrease in Biofilm

formation in most bacterial isolates for both agents. For Ciprofloxacin, the isolate K1, which changes from strong to Non-forming producer, while the isolates K3, K4, K6, K8, K10, and K11 change to moderate producers, isolates K9, K12, and K13 change to Non- Biofilm producers.

Table 4. The effect of Sub-MIC antibiotics on biofilms formation by *K. pneumoniae*

No. of the isolates	After Ciprofloxacin treatment			After Trimethoprim-sulfamethoxazole treatment		
	Sub MIC	Absorbency at 630 nm	Biofilms formation	Sub MIC	Absorbency at 630 nm	Biofilms formation
<i>K. pneumoniae</i> 1	32	0.082	Non-biofilm producer	512	0.077	Moderately
<i>K. pneumoniae</i> 2	4	0.065	Moderately	512	0.047	Non-biofilm producer
<i>K. pneumoniae</i> 3	32	0.087	Moderately	512	0.078	Moderately
<i>K. pneumoniae</i> 4	16	0.076	Moderately	32	0.061	Moderately
<i>K. pneumoniae</i> 5	4	0.061	Moderately	32	0.077	Moderately
<i>K. pneumoniae</i> 6	32	0.070	Moderately	512	0.068	Moderately
<i>K. pneumoniae</i> 7	32	0.060	Moderately	256	0.048	Non-biofilm producer
<i>K. pneumoniae</i> 8	16	0.073	Moderately	4	0.040	Non-biofilm producer
<i>K. pneumoniae</i> 9	32	0.044	Non-biofilm producer	512	0.049	Non-biofilm producer
<i>K. pneumoniae</i> 10	32	0.069	Moderately	256	0.059	weak
<i>K. pneumoniae</i> 11	32	0.060	Moderately	256	0.048	Non-biofilm producer
<i>K. pneumoniae</i> 12	16	0.047	Non-biofilm producer	256	0.051	weak
<i>K. pneumoniae</i> 13	4	0.038	Non-biofilm producer	4	0.045	Non-biofilm producer

For Trimethoprim-Sulfamethoxazole, The isolates K2, K7, K9 and K13 change from moderate to Non -Biofilm producer, While isolates K1, K3, and K6 from strong change to moderately Biofilm producer and one isolate K8 change from strong to Non -Biofilm producer, K12 change from strong to weak.

Antibacterial activity of Aqueous and Alcoholic Extracts of M. parviflora on K. pneumoniae

In this study, The results illustrated in Table 5. Showed that Methanolic and Ethanolic extracts given a high effect against isolates of *K. pneumoniae* more than the effect of a Hot extract in this study. A hot aqueous extract had 11 mm effect on all isolates (Sensitive) at Concentration 200 mm, But at 50 or 100 was no effect. The extracts of Methanolic extracts of *M. parviflora* with inhibition diameters of 12 mm at Concentration of 50 mg/dl on isolates are Sensitive, 24 mm at 100 mg/dl and 28 mm at 200 mg/dl. The imbibition diameters of Ethanolic extract were recorded an effect, 16 mm at 50 mg/dl, 26 mm at 100 mg/dl and 30 mm at Concentration of 200 mg/dl .

Moreover, the ethanolic and methanolic extracts did not show any inhibition at the concentration of 50 mg/dl for the resistant isolates, compared to the concentrations of 100 mg/dl and 200 mg/dl, which showed the highest inhibition zones, Which it 24 mm, 28 mm, and 26 mm, 30 mm, for the Ethanolic and Methanolic extracts, respectively.

Table 5. The effect of *M. parviflora* Aqueous, Methanolic and Ethanolic extracts on biofilms formation by *K. pneumoniae*

Concentration mg/ml	Hot Aqueous Mean±SD		Methanolic extract Mean±SD		Ethanolic extract Mean±SD	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
50	•±•	•±•	•±•	12±°	•±•	17±^
100	•±•	•±•	21±•.6	24±0.7	20±0.5	26±0.7
200	•±•	11±0.3	25±0.5	28±0.8	24±0.6	30±1.0

0=No inhibition zone, SD=Standard Deviation

The results are presented in Table 6. Indicate that treatment with alcoholic extracts (ethanol and methanolic) led to a decrease in the formation of biofilms of *K. pneumoniae* isolates. Biofilm Formation was reduced in most isolates from strong to moderate, weak, and non-biofilm producing, indicating a strong effect of the extracts in inhibiting the formation of biofilms of these isolates. As follows. For methanolic extracts, the effect on 5 isolates changes to Non-producers of biofilm, K1, K6, K7, K8, and K13, While 5 isolates change to weak K2, K5, K9, K10 and K12 change to weak biofilm formation. 3 isolates ,K3, K4, and K11 change to moderately formation of biofilm. While for Ethanolic extract, 4 isolates change to Non-producer of biofilm K1, K6, K8, and K13, while 6 isolates change to weak K2, K5 ,K7, K9, K10, and K12. Finally 3 isolates, K3, K4, and K11 change to moderately.

Table 6. Effect of *M. parviflora* extracts(Sub-MIC) on biofilms formation by *K. pneumoniae*

Isolates No. 13	After Methanolic Treatment (Sub- MIC 100)		After Ethanolic Treatment (Sub- MIC 100)	
	Absorbency at 630 nm	Biofilms formation	Absorbency at 630 nm	Biofilms formation
<i>K. pneumoniae</i> 1	0.041	Non-biofilm producer	0.048	Non-biofilm producer
<i>K. pneumoniae</i> 2	0.053	Weak	0.055	Weak
<i>K. pneumoniae</i> 3	0.077	Moderately	0.070	Moderately
<i>K. pneumoniae</i> 4	0.078	Moderately	0.080	Moderately
<i>K. pneumoniae</i> 5	0.048	Weak	0.050	Weak
<i>K. pneumoniae</i> 6	0.045	Non-biofilm producer	0.041	Non-biofilm producer
<i>K. pneumoniae</i> 7	0.038	Non-biofilm producer	0.052	Weak
<i>K. pneumoniae</i> 8	0.030	Non-biofilm producer	0.045	Non-biofilm producer
<i>K. pneumoniae</i> 9	0.050	Weak	0.047	Weak
<i>K. pneumoniae</i> 10	0.052	Weak	0.051	weak
<i>K. pneumoniae</i> 11	0.077	Moderately	0.078	Moderately
<i>K. pneumoniae</i> 12	0.056	Weak	0.053	weak
<i>K. pneumoniae</i> 13	0.049	Non-biofilm producer	0.044	Non-biofilm producer

DISCUSSION

Aqueous and alcoholic extracts of *M. parviflora* yielded varying activity levels against multidrug-resistant strains of *K. pneumoniae*, ranging from inactivity to potent growth inhibition. Alcoholic extracts generally showed significant growth inhibition against the tested bacterial strains. At concentrations of (100,200) mg/ml, methanolic and ethanolic extracts were found to exhibit distinct activity against biofilm formation in the tested isolates.

Biofilm is one of the important factors in the virulence of bacterial isolates and has major role in Pathogenesis, bacterial isolates Biofilm forming can withstand difficult environmental conditions, as it protects bacteria from attack by antibodies and their decomposition by the immune system, phagocytosis, and elimination through the endocytic cells, This occurs within the Biofilm at a high level (Eze et al., 2018) .

The ability of bacterial isolates to form Biofilm was variable, and this was due to some factors affecting their ability, Such as temperature, type of surface to which the Biofilm adheres, physical interaction between the components, pH, etc (Chérif et al., 2016). In study by Wang et al., (2019), Showed that the reasons for the difference in the results of the study with other studies is due to several reasons that may effect on the results, Such as the difference in the composition of some medium such as Nutrient broth and Typtic Soy Agar (TSA), Microbial Concentration, Incubation period (24hours -48hours), The density of cells inside Biofilm as the incubation period increases, In addition to Concentration of the dye used in experiment. There may be several factors affecting the Biofilm density among the different isolates in this study, which affect the ability of the isolates to build Biofilm. In addition to the difference in the quantity and quality of sensing quorum signals produced by each isolate which play an important and major role.

K. pneumoniae have multiple resistance mechanisms to beta-lactam antibiotics for inhibit actions, including have Efflux pump (AcrAB) system that leads to the removal of antibiotics from the bacterial cell and Deletion of porin genes (OmpK36), It prevents antibiotic entry into the cell (Bush and Bradford, 2020;Saxenborn et al., 2021). And thus a Mutation occurs in outer membrane proteins (OMPs) leading to changes in OMP structure and reduced antibiotic entry .

Resistance of bacterial isolates to Doxycycline was 76.7%, illustrated in Figure 2. The increase resistance to class is the wrong use and low cost, which has led to a high in the rate of resistance in the past few years. It is also believed that widespread and consistent use of Doxycycline enabled bacteria to form resistance determinations that can protect the ribosome binding site, and thus reduce the binding of the drug to the active site, while resistance to the Sulfonamide Class was 69.2%. Quinolones inhibit bacterial DNA synthesis by inhibiting the enzymes Topoisomerase IV and Topoisomerase II (DNA gyrase). This prevents DNA replication and repair, leading to bacterial death. Thus, it is widely usage for treatment of Urinary tract infections and respiratory diseases (Tang and Zhao, 2023) .

The study demonstrated that the highest rate of isolates resistance to Ampicillin in this study. The reason for high resistance to it, is that all the bacterial isolates under study are capable of secreting the chromosomally encoded Beta Lactamase (Navon-Venezia et al., 2017). Furthermore, isolates of *K. pneumoniae* showed moderate resistance to Azithromycin, The reason for resistance most of isolates possessing *erm* genes that inhibit interaction Azithromycin with the ribosome, Thus Inhibition of

protein synthesis (Liu et al., 2022)

These results of resistance to Imipenem and Meropenem are due to less frequent usage of these agents compared to other agents used to treat various infections. Any increase in the emergence of resistance to Carbapenems Class requires continuous and careful monitoring among hospitalized patients, as well as among immune-compromised patients. Due to emergenc in resistance of bacteria to the chass of antibiotics, because it produce enzymes degrade Carbapenem Class called carbapenemases. *K. Pneumoniae* is one the most important organisms that have attracted public health attention due to the clinical importance they have acquired due to their important role in spreading their antibiotic resistance agents to other types of bacteria present in the same environment (Nirwati et al., 2019). *K. pneumoniae* isolates have three mechanisms to resistance to Amikacin by reducing in permeability of membrane of bacteria to it, Chromosomal mutation in target site of protein encoded by gens and modification of enzyme that modify antibiotics. Relative difference in resistance patterns in *K. Pneumoniae* to various antibiotics. It can happen for several reasons including, geographical differences in samples, diversity in site of infection, size of the study's sample and the patient's infections. But in general, *K. Pneumoniae* is a multi-resistant, ready-to-infect pathogen with a high level of resistance to Antibiotics (Abdel-Rhman, 2020).

The current study showed the results illustrated in Table 4. It was recorded that Sub MIC to Ciprofloxacin and Trimethoprim-Sulfunmethoxazole after incubation for 24hrs, Biofilm density is reduced in most of the studied isolates. The difference in the results of this study and others may be due to the types of isolates in each study, in addition to the genetic composition and the condition in the laboratory (Coughlan et al., 2016). Microtiter Plate Method is the best method for calculating the MIC of antibacterial agents, because it saves time and cost, it is easy to use, and has high accuracy in the results.

In Table 4, as shown in the results of the current study to demonstrate the effect of the Sub-MIC on biofilm formation after treatment with Ciprofloxacin, The average absorbance value was recorded (0.063 ± 0.01)mg/ml at a wavelength of 630 nm, Where it was clear that there no significant difference between the concentrations. Among 13 isolates, 9 isolates were classified at 76.9% as Moderately biofilm, while 4 isolates at 23.1% were Non-biofilm producer, where a trend of increasing biofilm formation was observed with increasing concentration, and the concentration of 32 mg/ml recorded

the highest average absorbance of (0.015 ± 0.067) mg/ml, Meaning that the MIC of the antibiotic does not significantly affect (prevent biofilm formation) the ability of the isolates to form biofilm under the experimental conditions.

The results also showed that the effect of the Trimethoprim-Sulfamethoxazole concentration was different on the formation of the biofilm Table 4, as there was a clear variation in the response of the isolates to the formation of the biofilm with a significant effect at $p < 0.005$. All the isolates at the concentration of 4 mg/ml were not forming biofilm (100%), At the concentration of 32 mg/ml all the isolates were Moderately biofilm formation (100%), while the remaining concentrations showed a different distribution among the isolates, And this indicates the presence of a different response to the concentrations. The isolates were distributed into three groups: 6 isolates (46.1%) were Non-Biofilm-producers, 5 isolates (38.5%) were weak, and the remaining 2 isolates (15.4%) were Moderately . The average absorbance value was (0.058 ± 0.013) mg/ml at a wavelength of 630 nm, where there was a significant difference between the concentrations.

The overuse of antibiotics has led to the emergence of resistant microorganisms. Due to the rapid spread and multiplication of bacteria, resistance genes can be transferred to non-resistant bacteria, resulting in antibiotic resistance (Mancus et al., 2021). This leads to uncontrolled resistance. In addition, biofilm-forming bacteria are increasingly resistant to antimicrobial drugs, making treatment more complex and highlighting the need for new therapeutic strategies .

Antibacterial Activity of M. parviflora against isolates

The results presented in Table 5, indicate that Methanolic and Ethanolic extracts showed significant efficacy against biofilm formation of K. pneumoniae isolates, with this efficacy being higher than that of antibiotics. The study also suggests that the ability of K. pneumoniae to produce biofilms makes it more vulnerable to developing resistance to commonly used antibiotics, highlighting the need for effective therapeutic alternatives such as Methanolic and Ethanolic extracts of plants that are effective against various pathogens.

In Table 5, as documented, the study results showed a clear variation in the antimicrobial activity of the aqueous and alcoholic extracts of M. parviflora against K. pneumoniae isolates. At a concentration of 50 mg/ml, the results showed only low activity compared to the other concentrations. The

methanolic and ethanolic extracts recorded activity against sensitive isolates with an inhibition zone of (12 ± 0.5) mm and (17 ± 0.8) mm, respectively, while the other groups did not show any antimicrobial activity. This is because water is polar and only extracts polar active compounds such as sugars, steroids and amino acids, which showed only weak activity against the sensitive and resistant isolates under study. With increasing the concentration of the extracts to 100 mg/ml, the activity showed a clear improvement against the sensitive and resistant bacterial isolates. The results recorded four inhibition zones against the isolates, showing a significant difference $p < 0.005$. At a concentration of 200 mg/ml, all extracts were effective against bacterial isolates. The results showed a direct relationship between increasing concentration and the expansion of the inhibition zone, with a significant difference, $p < 0.005$. The results showed significant differences between the types of extracts. At a concentration of 100 mg/ml, the t-test for comparisons between groups showed that the group representing the effectiveness of the ethanolic extract (26 ± 0.7) mm significantly outperformed the rest of the groups.

The study results recorded a clear variation in the ability of biofilm formation of the isolates under study. There were significant differences between the groups $p < 0.005$. The isolates were also distributed into three categories after treatment with Methanolic extract. 5 isolates were non-biofilm-producers at 35.7%, 6 isolates were weak at 42.9%, while 3 isolates were moderately at 21.4%. It also showed that the highest absorbance values were for the moderately group (0.076 ± 0.005) , then the weak group (0.053 ± 0.004) , then the Non-Biofilm producer group (0.046 ± 0.003) .

Also, in Table 6 the current results showed that biofilm formation at a wavelength of 630 nm showed a variation in the ability of bacterial isolates to form biofilms after treatment with the Ethanolic extracts. There were significant differences, $p < 0.005$. The isolates were distributed into three groups: 5 isolates 38.5% were non-film producer, 5 isolates 38.5% were weak, and the remaining 3 isolates 23% were moderately. Moderate gave the highest absorbance value (0.077 ± 0.01) , then the weak (0.05 ± 0.01) , the non-biofilm producers (0.041 ± 0.008) , and this indicates the presence of significant differences $p < 0.005$. None of the isolates under study was found to be strong biofilm-forming after treatment with *M. parviflora* extracts.

In A Study conducted by Ramírez-Serrano et al., (2019). showed that the chloroform extract of *M. parviflora* was found to have antibacterial activity against *E. coli* and *Staph. aureus* with MIC of 20

mg/ml. Whereas, the Ethanol extract showed antibacterial activity against *Staphy. aureus* only with MIC of 15 mg/ml. These results suggest that *Staph. aureus* was more sensitive to the Ethanolic extract than the Chloroform extract. It is believed that the Ethanol solvent has a greater extraction capacity, which may yield more active components responsible for the antibacterial activity against *Staph. aureus*.

Previous studies, after conducting chemical analysis of the plants, It have shown the presence of many compounds with biological and therapeutic activity, Phenolic compounds are one of the most important metabolites in the plant (Derong et al., 2016). It has been shown to have anti-cancer and anti-inflammatory activity (Uddin et al., 2014). Antioxidant properties have been described for plants with high phenolic compounds, which are represented by phenolic acids, flavonoids, and tocophenols (Tana et al., 2016; Joshi et al., 2013). The effect of these compounds is due to their ability to dissolve and form complexes with the bacterial cell wall and interact with extracellular protons (Lyubchyk et al., 2019). Saponins have been shown to have anti-inflammatory activity, as they are characterized by their ability to form foam in aqueous solution and have hemolytic activity (Agidew, 2022). The plant also contains Steroids and alkaloids that have anti-inflammatory activity. The antibacterial activity of plant extracts is likely due to secondary metabolites such as Saponins, Alkaloids, Phenols, Sugars, and Terpenoids, which have activity alone or in combination with each other to be potential defense mechanisms of great importance against various types of pathogens (Teoh, 2015). These compounds have antibacterial activity that inhibits the cell wall construction of microorganisms, which allows for effectiveness against these organisms, by depleting the energy of organisms and accumulating these compounds in the plasma membrane of organisms (Agidew, 2022; Raquel, 2007).

Although the results of the current study demonstrated clear and effective activity of *M. parviflora* extracts against biofilm formation in vitro, the study's limitations, including pharmacokinetics, toxicity, and host interactions, mean that in vitro models cannot be considered due to the biological complexity of infection in vivo. Therefore, to confirm the validity of in vivo study results until the therapeutic physiological conditions are known, these studies will assist future studies to evaluate safety and therapeutic efficacy, as well as any consideration of potential applications.

CONCLUSIONS

The antimicrobial activity of plant extracts represents a promising therapeutic option given the

development of antibiotic resistance and health risks associated with synthetic preservatives. *M. parviflora* is emerging as a promising medicinal herb, containing bioactive compounds capable of affecting the cellular functions of microbes. The study confirm that aqueous and alcoholic extracts of *M. parviflora* have a significant ability to inhibit biofilm formation in pathogenic bacteria. Although further clinical studies and in vivo experiments are needed, these findings open the way for the development of new pharmaceuticals and functional food products. We recommend conducting future studies that focus on identifying the active compounds responsible for the antimicrobial activity, testing their safety, determining their effectiveness in animal models, and studying the synergies between antibiotics and the active compounds of plant extracts. This will be an advanced step towards translating the results of the current study into various effective clinical treatments that can be applied in the future.

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Ethical clearance :The study confirms its adherence to ethical standards, as ethical approval was obtained from the Diyala Health Directorate under code number 67595 on November 27, 2023. Participants and healthcare workers were informed of the study objectives, and patients were informed of the benefits and the absence of health concerns associated with the study. Participants participated voluntarily with the right to withdraw at any time without adverse effects on their treatment or health. Precautions were taken to maintain data confidentiality through coding. Participants were informed that the data would be used only for study purposes, and verbal consent was obtained from them .

Conflicts of interest: Nil

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