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Antibacterial Activity of *Capsicum annum* L. Juice Against *Klebsiella pneumonia* Isolated from Respiratory Tract Infections

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ABSTRACT

Aims *Klebsiella pneumoniae* can be defined as one of the virulent pathogens with a high level of antibiotic resistance. The goal of the presented work was to see if *Capsicum annuum* L. juice has antibacterial activity against *K. pneumonia*, a bacteria seen in respiratory tract infections.
Materials & Methods Fifty respiratory tract infections have been collected that include such bacterium's resistance to some antibiotics, the qualitative detection of *C. annuum* L. juice effective groups, the minimal inhibitory concentration of *C. annuum* L. juice, and the inhibition activity against the growth of *K. pneumoniae*, and its cytotoxicity was studied.
Findings The cultural results showed that 12 (24%) isolates were related to *K. pneumonia,* and the associated virulence factors with *K. pneumonia* pathogenicity are capsule, hemolysin, urease, biofilm production, and β-lactamase production. All isolates showed resistance to 100% Ampicillin and indicated that juice contained high percentages of active compounds. The minimal inhibitory concentration value of *C. annuum* L. was 6.25mg/mL for the bacterial isolates related to *K. pneumoniae.* There has been a considerable difference at p≤0.050, while the cytotoxicity shows that Juice *C. annuum* L. has no cytotoxic activity against human red blood cells.
Conclusion Antibacterial activity of *C. annuum* L. juice increases with the increase of its concentration, and it is possible to use it as an adjunct treatment to eliminate or reduce the growth of some pathogens.

Keywords Anti-Bacterial Agents; *Klebsiella pneumoniae* beta-lactamase CTX-M-2; Respiraa tory Tract Infections

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Introduction

Klebsiella spp. are Gram-negative, bacilli, nonmotile, facultative anaerobe with a capsule belonging to the Enterobacteriaceae family. The infection of *Klebsiella* spp. is difficult to treat using an antibiotic, especially pneumonia [1]. The bacteria are present on the skin, pharynx, and digestive tract, and their presence in soil and water renders their existence in fruits and vegetables. They are an opportunistic pathogen; they cause various infections in diabetics, chronic lung patients, immunocompromised people, and nosocomial infections [2]. The species infections are widespread in Eastern Europe, North Asia, Central Africa, and Latin America; hence, they are widespread [3]. This bacterium was first isolated by the scientist Friedlander from pneumonia in 1882 and caused endemic infections in hospitals during the 1950s [4]. These bacteria are widespread and isolated from different habitats such as the human body, animals, sewage, soil, lakes, saltwater, and freshwater. Generally, they are opportunistic pathogens to humans and animals [5]. *Klebsiella* strains have developed numerous resistances to antibiotics as a result of the overuse of unusually broad-spectrum antibiotics, and one of the causes can be their production of broad-spectrum β-Lactamase enzymes. *K. pneumoniae* is an opportunistic pathogen found in individuals suffering from immunosuppressive disease and those with urinary tract infections [6]. It is resistant to phagocytosis due to its capsule [7]. The virulence factors of *Klebsiella* bacteria are the antigen of the capsule [8], which pose as its virulence factors besides lipopolysaccharide (LPS), Bacteriocin [9], and the formation of Biofilms [10]. Bacterial Haemolysin, a toxin that damages the cellular membrane related to RBCs, resulting in their decomposition and bacteremia [11], is another virulence factor. Urease is produced via several bacteria which result in UTIs, including Klebsiella, Pseudomonas, and Proteus, according to recent research [12]. Bacteriocins aren't direct virulence factors, yet they do increase the possibility of strains competing in producing strains [13]. *K. pneumoniae* produces Bacteriocin called Klebocin [14]. Bacteriocins inhibit the growth of other bacteria, either acting as a Bacteriostatic [15] or a Bactericidal [16].

Medicinal plants: The plant produces secondary chemical compounds from the primary metabolites during its life processes, which are found inside the plant tissues in the form of three main groups: Phenolic, Alkaloid, and terpenoid compounds as defensive materials by which it protects itself from micro-organisms, insects, and nematodes [17]. Medicinal plants have been used for various purposes, such as to treat human diseases, such as Antioxidants and anti-cancer. Besides, medicinal plants are widely used in various industries such as preservatives, flavorings, and delicious appetizers [18]. Plant extracts have been used as agents in fungal and bacterial infections because they are inexpensive, safe to use, and abundant [19]. Authors studied the relationship between a plant's components and its pharmaceutical efficacy by utilizing these herbs as medicinal sources and developing them based on local botanical floral [20]. *C. annuum* L. comprises many varieties with increased nutritional value and a significant source of vitamins E, C, and A. It is commonly used as a flavoring and natural colorant for food, besides other medicinal benefits such as Anti-inflammatory, Anti-allergenic, and Anti-carcinogenic. The potential of mature red peppers, the pimento, to reduce cancer risk has been documented [21, 22]. It belongs to the Solanaceae family, with 27–30 known species, of which only five are domesticated, i.e., *Capsicum annum*, *Capsicum frutescens*, *Capsicum pubescens*, *Capsicum baccatum,* and *Capsicum Chinese* [22].

The current study aims to isolate *K. pneumoniae* isolated from RTIs and identify the epidemiology of these bacteria isolated from adult patients in Karbala city. Besides different virulence factors of this bacterium and its resistance to some antibiotics, Evaluation of cytotoxicity and Antibacterial Activity of *C. annuum* L. juice were also assessed and compared to antibiotics.

Materials and Methods

A total of 50 samples of sputum have been obtained from both male and female adult patients with RTIs. The bacterial isolates were diagnosed by studying the general culture characteristics of colonies in MacConkey and blood agar. Next, the apparent colonial forms were studied and determined based on texture, color, shape, and size, as well as noting other general characteristics such as lactose fermentation and the presence of blood hemolysis on blood agar [23]. Microscopic examination of bacterial cells was also carried out by staining them with a Gram stain and studying the oil immersion technique under the microscope. Also, the assessments included the interaction of cells with the stain, Note cell morphology, and its grouping. Biochemical tests were also conducted, i.e., Catalase, Motility, Oxidase, Methyl Red, Indole, Citrate Utilisation, Voges-Proskauer, and Urease tests. The API 20 E kit and Vitek 2 system were used to confirm the diagnosis of bacteria. Investigation of Virulence Factors in *K. pneumonia* was based on the following:

- Capsule: using the negative staining method [24].
- Hemolytic enzyme test: Bacterial isolates were cultured to be tested for their ability to produce the Hemolysin enzyme [25].
- Urease enzyme test [25].
- Biofilm formation with Congo-red method [25, 26].

The rapid iodometric standardized iodine method [27] has been utilized to investigate the capability of isolates understudy to create β-lactamase. The Kirby-Bauer test was used to test for susceptibility to antibiotics [28]. The juice of *C. annuum* L. was extracted using an electric extractor, followed by a centrifuge process at 3000 rpm for 5 mins. Also, the solution was filtered with the use of Whatman No one filter paper, while the filtrate has been sterilized with the use of filter 0.22µm. It was then concentrated using the rotary evaporator under vacuum pressure at 40 °C to obtain the juice of *C. annuum* L. The thick liquid was then placed in an oven at 37°C to acquire the dry juice. A series of half-dilution (1.56, 3.12, 6.25, 12.5, 25, and 50mg/mL) was applied to the *C. annuum* L. powder [29]. Qualitative chemical tests were conducted to reveal some of the active groups of *C. annuum* L. juice, which included the detection of Glycosides, Phenols, Tannins, Saponins, Resins, Alkaloids, Flavones, and pH [30]. The broth dilution technique [31], which depends on the turbidity of bacterial growth, was used to prepare a series of *C. annuum* L. juice to determine the minimal inhibitory concentration (MIC) [32]. For the antibacterial activity test of *C. annuum* L. juice against *K. pneumoniae* isolated from respiratory tract infection, a good diffusion technique was used and included based on the method [33]. Also, for evaluation of the cytotoxicity of *C. annuum* L. juice, human red blood cells were used to calculate the cytotoxicity of *C. annuum* L. juice [34]. The resistance of the isolates of *K. pneumoniae* used in studying 7 of the various Antibiotics was determined based on the inhibition area's measured diameter and compared with a previous study [35].

The results have been statistically analyzed with the use of one and two variance analyses and the Duncan multiple range analysis tests along with the descriptive statistics and the standard deviation, according to the statistical computer system (SPSS 24) [36].

Findings

- Isolation and Diagnosis of *K. pneumoniae*:

We obtained 12 (24%) isolates of the bacteria belonging to *K. pneumoniae* from a total of 50 samples that have been obtained from respiratory tract infections. It is identified by the Gram-negative bacilli, non-motile, and lactose fermented, non-spore-forming, and surrounded by a capsule with a single or paired shape or short chains. The culture characteristics and biochemical tests were conducted to distinguish them from the remaining species.

- Investigation for Virulence Factors:

The current study included an investigation of many virulence factors associated with *K. pneumoniae* pathologies in Table 1:

- Capsule: Results have shown that under the microscope, all (100%) isolates under study contained a capsule, and the cells appeared in the form of bacilli surrounded by a halo in the nigrosine stain (black stain). Similarly, the laboratory examination showed that all isolates contain a capsule.
- Haemolysin Production: The results exhibited that all *K. pneumoniae* isolates did not produce hemolysis in blood agar, indicating the absence of Hemolysin. Haemolysin is an important virulence factor for various Gram-negative and -positive bacteria and contributes to the occurrence of pathogenicity, and there may be several possibilities for the inability of this bacterium to produce Hemolysin.
- Urease Production: Results have shown that all (100%) *K. pneumoniae* isolates could produce urease.
- Biofilm Production: Results have shown that all (100%) isolates could produce biofilm, and all colonies appeared black with a dry crystalline density, indicating a positive result.

Table 1) The virulence factors present in the bacterial isolates belonging to *K. pneumoniae*

Isolates No.	Capsule	Hemolysin production	Urease production	Biofilm production
K.pn1	+	-	+	+
K.pn2	+	-	+	+
K.pn3	+	-	+	+
K.pn4	+	-	+	+
K.pn5	+	-	+	+
K.pn6	+	-	+	+
K.pn7	+	-	+	+
K.pn8	+	-	+	+
K.pn9	+	-	+	+
K.pn10	+	-	+	+
K.pn11	+	-	+	+
K.pn12	+	-	+	+

-Investigate of β -Lactamase Production:

This study showed that 9 (75%) *K. pneumoniae* isolates provided a positive result for the test. However, the difference in the time of positive reaction is significant, ranging from a few seconds to 2 minutes.

-Resistance of Bacteria to Antibiotics:

Table 2 shows all (100%) isolates showing complete resistance to Ampicillin.

The results of qualitative detection of the effective groups of *C. annuum* L. juice showed that it contains Glycosides, Saponins, Tannins, Flavonoids, Alkaloids, and Resins, with a pH of 5.1 in Table 3.

- Determining MIC of *C. annuum* L. Juice: The MIC value of the bacterial isolates belonging to *K. pneumoniae* used in the study against *C. annuum* L. juice was 6.25mg/mL.

Table 2) The percentage of *K. pneumoniae* isolates resistance in the study to some Antibiotics

Isolates No.	AMP	TE	GM	CIP	AK	C	IMP
K.pn1	R	R	R	R	R	R	S
K.pn2	R	R	R	R	R	R	S
K.pn3	R	R	R	R	R	S	S
K.pn4	R	S	R	R	R	S	S
K.pn5	R	R	R	S	S	R	S
K.pn6	R	R	S	R	R	R	S
K.pn7	R	S	R	R	S	S	S
K.pn8	R	R	R	R	S	R	S
K.pn9	R	R	R	S	R	S	S
K.pn10	R	R	S	R	S	R	S
K.pn11	R	R	S	R	R	S	S
K.pn12	R	R	R	R	R	R	S

R: resistance; S: Sensitive; Ampicillin (AMP), Tetracycline (TE), Gentamycin (GM), Ciprofloxacin (CIP), Amikacin (AK), Chloramphenicol (C), Imipenem (IMP)

Table 3) Results of the qualitative chemical detection of some active compounds and the pH Estimation of *C. annuum* L.

Active groups	Reagent	Detection guide	Results
Glycosides	Benedict's Reagent	Red precipitate	+++
	Fehlank's Reagent	Red precipitate	**+ ++**
Saponins	Mercuric Chloride Reagent	White precipitate	++
	Shake the juice	Dense foam	++
Tannins	Lead acetate Reagent 1%	White precipitate	++
	Ferric Chloride Reagent 1%	Bluish-green color	**++**
Flavonoids	Potassium hydroxide	Yellow precipitate	+
Alkaloids	Meyer's detector	Brown precipitate	+
Resins	Ethanol / distilled water	Turbidity	+
pH	/	/	5.1

The sign (+) indicates positive detection by 0.1% of the weight of sample that has been taken for analysis, while (++) 0.2% of the weight of sample that has been taken for analysis, and so on. And according to the color gamut in one model.

A total of 12 bacterial isolates from Respiratory Tract Infections, confirmed and used to assess *C. annuum* L. juice efficacy against the growth of *K. pneumoniae* bacteria using the well diffusion method in Table 4. Four different *C. annuum* L. juice concentrations were used, i.e., 50, 25, 12.50, and 6.25mg/mL and the fifth well for control. The four concentrations were used to compare the inhibition efficiency of each concentration. The concentration of 50mg/mL of *C. annuum* L. juice affected the bacterial isolate the most, with the highest inhibition diameter of 33mm and the minimum inhibition diameter of 25mm. The 25mg/mL concentration exhibited the highest inhibition diameter at 30 mm and the lowest at 18 mm. In contrast, 12.5mg/mL concentration gave the maximum inhibition diameter of 28mm and the lowest inhibition diameter of 16 mm. Finally, the 6.25mg/mL concentration showed the highest inhibition diameter of 25mm and the lowest inhibition diameter of 12mm compared to the control factor containing sterile distilled water.

Table 4) Diameter of the inhibition zones in (mm) for different concentrations of *C. annuum* L. juice against the growth of *K. pneumoniae* Isolated from Respiratory Tract Infections

Isolates No.	Concentrations (mg/mL) 6.25		12.50		25		50		p-value
	SD.	Mean	SD.	Mean	SD.	Mean	SD.	Mean	
K.pn1	1 d C	22	1 c D	24	1 b D	27	1 a CD	30	0.01
K.pn2	1.73 c D	25	2 b E	28	1 ab E	30	1 a D	32	0.003
K.pn3	2 c A	14	2 b ABC	18	1 b B	21	2 a B	26	0.01
K.pn4	2 c A	14	1.73 bc AB	17	1.73 b AB	20	1.73 a B	25	0.01
K.pn5	1 d C	22	1 c D	24	1 b D	27	1 a CD	30	0.01
K.pn6	1.73 d B	17	1 c CD	21	1 b C	24	2 a BC	28	0.01
K.pn7	1 c CD	24	2 b E	28	1 ab E	30	2 a D	32	0.002
K.pn8	2 d A	12	2 c A	16	1.73 b AB	20	1.73 a B	25	0.01
K.pn9	1 c A	12	2 b A	16	2 b A	18	1.53 a A	21.33	0.001
K.pn10	1.73 c D	25	2 b E	28	1 b E	30	1 a D	33	0.001
K.pn11	2 c A	14	1.73 bc AB	17	1.73 b AB	20	1.73 a B	25	0.01
K.pn12	1 d B	17	1.73 c BC	20	1 b C	24	2 A BC	28	0.01
p-value	0.01		0.01		0.01		0.01		

- Similar small letters indicate the absence of significant differences. Meanwhile, the different letters indicate significant differences between the concentration and bacterial isolation at the p≤0.05 probability level.
- Similar capital letters indicate the absence of significant differences. As for the difference, there have been significant differences at the p≤0.05 probability level between bacterial isolates and each concentration.

Statistical results have shown significant differences at p≤0.050 between isolates of one concentration or between concentrations for one isolate, and the results also did not show significant differences for the interaction (isolation + concentration).

The best rate of inhibition diameters was noted in the 50 mg/mL concentration. The increase in the diameters of the inhibition areas was compared to the increase in juice concentration due to its high inhibition activity against the pathogenic bacteria

under study. The inhibitory effect of *C. annuum* L. juice can be attributed to its active inhibitory compounds.

- Cytotoxicity of *C. annuum* L. Juice:

C. annuum L. juice did not show any cytotoxicity against Human Red Blood Cells. This is indicated by the absence of hemolysis in the series of concentrations prepared from *C. annuum* L. juice, as shown in Table 5.

Table 5) Cytotoxicity of *Capsicum annuum* L. juice

Material used	Hemolysis
***Capsicum annuum* L. juice** (mg/mL)	
50	-
25	-
12.50	-
6.25	-
Physiological salt solution	-
Tab water	+

(+): Hemolysis; (-): Non-hemolysis

Discussion

The microscopic, culture and biochemical tests were conducted according to a previous study [5]. The *K. pneumoniae* isolates were confirmed using the API 20 E and Vitek 2 system, characterized by ease, speed, and accuracy. Statistics indicate that *K. pneumoniae* causes 3% of epidemic diseases, and this bacterium causes 14% of bacteraemia cases [37]. The diseases caused by this bacterium are diseases acquired in hospitals, including inflammation of the urinary and genital tract, surgical wounds, and meninges [38-40]. *K. pneumoniae* is the second cause of bacteraemia and fatal septicaemia in infants and newborns, and acute illnesses, especially in immunocompromised people due to diabetes, chronic heart disease, and pulmonary vasoconstriction, especially in older persons and newborn babies. Most *Klebsiella* infections in hospitals are highly fatal if not treated properly [8]. This bacterium also causes otitis media infections, soft mucous membranes, nasal necrosis, and lung infections [41]. Of virulence factors, Authors [42, 43] reported that the K1 serotype found in *K. pneumoniae* capsules is responsible for the hospital-acquired infections with (14- 23.4%).

The capability of *K. pneumoniae* to produce haemolysin in blood agar [44]. The Authors also proved [45] that a mutation in the *hns* gene in the *K. pneumoniae* strain showed the ability of bacteria to show activity in haemolysin production. The significance of the urease enzyme also comes from its capability to analyze urea to ammonia NH4 and carbonic acid H2CO3, a nickel-containing crystalline enzyme and causes the formation of stones in the kidneys, pelvic nephritis, causes encephalopathy due to ammonia (known as encephalopathy ammonia), and hepatic coma [46].

The ability of *K. pneumoniae* to produce biofilm [47], was assessed using Congo red method, and the production rate of its isolates for the biofilm reached 83%. Biofilm production is affected by the density of the bacterial culture and is more sensitive to using Congo red dye, which is accountable for dyeing the polysaccharide layer, the biofilm's basis [25]. Several environmental factors can impact the mucous layer's production with the use of such an approach, including temperature, oxygen, and other conditions, which could provide different results [48]. The bacteria that grow in the biofilm are tolerant of many antibiotics and are resistant to both opsonization, phagocytosis, different environmental conditions, and resistant to selective pressures [49]. The presence of micro-organisms that make up the biofilm increases their resistance to antibiotics about 1000 times, and biofilm-producing bacteria are accountable for diseases and infections which are hard to treat due to the difficulty and restriction of antibiotic penetration [50].

K. pneumoniae can be defined as one of the nosocomial pathogens that are frequently linked to hospital outbreaks and have a high proclivity for antimicrobial resistance to β-lactamase antibiotics and a variety of other antibiotic classes. Various factors contribute to the Gram-negative bacteria's effective proliferation, infection, and transmissions, such as environmental factors, host factors, virulence factors, and many antibiotic resistance mechanisms. The effective spread and development of antibiotic resistance in increasingly frequent beta-lactamase-producing *K. pneumoniae* bacterium has resulted in bad treatment outcomes and limited therapy alternatives [51].

We note the resistance of *K. pneumoniae* to antibiotics in the presented work. The authors agree about the sensitivity of the intestinal family bacteria [52], including *K. pneumoniae*, to this antibiotic, as the rate of bacterial sensitivity reached 100%. The results agree with the study conducted by other authors [53]. It was proved that all *K. pneumoniae* isolates produce ESBLs, and not producing this enzyme were 100% sensitive to Imipenem. The changes in the protein openings of the outer membrane, including the loss of OMPK36, conferring *K. pneumoniae* resistance to this antibiotic [54].The resistance of the intestinal family among them, *K. pneumoniae* to anti-β-lactam is because of its capability for producing β-lactamase enzymes and its resistance by several mechanisms, including decreasing the antibiotic's permeability into the cell, also examining the antibiotics through β -lactamase, and the other decreasing the affinity to the enzyme Penicillin-Binding Proteins (PBP) [55]. The results are in line with previous studies [56]. as the rate of resistance to these antibiotics reached 84%. The plant's active compounds are also secondary metabolites that have defensive characteristics against micro-organisms and are considered toxic oxidants for micro-organisms [57]. Those agree with the results of many works showing that the study plant contains many active compounds [58]. The

effect of concentrated and dried plant juices against all bacterial species has been done by a similar mechanism to the action of some antibacterial drugs, as they may inhibit the development of cell walls, especially in Gram-positive bacterial strains [59]. The reason for the growth inhibition might be due to the capability of compounds to interact with the proteins of the cytoplasmic membrane, causing a change in the membrane permeability. It might lead to a defect in the respiratory and primary metabolic activities and impede the oxidative phosphorylation process and the electron transmission chain during the cell respiration process [60]. *C. annuum* L. juice was distinguished by its high content of these compounds (Glycosides, Saponins, Tannins, Flavonoids, Alkaloids, and Resins) in varying proportions and contained active groups, rendering its high inhibitory effect against the bacterial isolates under study. Whereas the MIC value of *C. annuum* L. juice depended on the concentration used and its containment of the active compounds, whereby the lower MIC of the extract was due to its containment of the active compounds and the extracts containing active substances with low molecular weights had a more inhibitory effect. The difference in the sensitivity of the bacterial isolates understudy to *C. annuum* L. juice is due to the nature of the bacteria itself in terms of composition and thickness of the cell wall and lipids and protein content. The inhibitory effect of *C. annuum* L. juice can be attributed to its high inhibitory efficacy against the pathogenic bacteria understudy, as it contains effective inhibitory compounds. We note that the juice of *C. annuum* L. did not cause any cytotoxicity to human erythrocytes. This indicates the absence of hemolysis in all concentration series prepared from the juice. *C. annuum* L., with all its varieties, is a high nutritional value vegetable and an important source of vitamins E, C, A, and many antioxidants and anti-allergenic substances.

Ural sources as an adjunctive treatment to eliminate or limit the growth of some pathogens and reduce the side effects of the antibiotics used at present that have severe effects on patients.

Conclusion

The Antibacterial activity of *C. annuum* L juice increases with the increase in its concentration. This is due to its active inhibitory compounds inhibiting the bacteria's growth by affecting the bacterial cell wall. Although a high inhibitory efficacy was noted compared to the antibiotics used in the study, this study also encourages the use of effective compounds isolated from nat.

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