

# Evaluation and quantification of the inhibition of biofilm and planktonic forms of *Klebsiella pneumoniae* by the polyphenolic extract of *Pulicaria crispa*

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## ABSTRACT

Analyses of polyphenolic plant extracts have shown significant results when used to control different pathogens. Many of these pathogens are responsible for different infections causing significant public health problems. This work aims basically to determine the efficiency of polyphenolic extract of *Pulicaria crispa* to prevent biofilm formation by *Klebsiella pneumoniae*. Strains were identified by their biochemical characters and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *P. crispa* is a Saharan plant used to extract polyphenols to assess their inhibitory action against *K. pneumoniae* development and biofilm forming. High-performance liquid chromatography revealed quercetin as the most important component of the polyphenolic extract. All strains are biofilm forming and are resistant to many antibiotics. The Minimal inhibitory concentrations of biofilm (MICBs) of the extract range from 0.21 mg gallic acid equivalent (GAE) to 3.40 mg GAE. The minimal inhibitory concentrations vary from 0.1 mg GAE to 0.425 mg GAE. Although many plant extracts have already shown their antimicrobial and antibiofilm activities, their application in clinical cases requires a long-term endeavor.

**Key words:** Antibiofilm, antimicrobial, *Klebsiella pneumoniae*, plant extract, respiratory pathogens

## INTRODUCTION

Besides the many advantages of antibiotics use in clinical practice, many drawbacks are caused by the intensive and the misuse of these powerful therapeutic agents. The emergence of a resistance phenomenon makes antibiotics less effective representing a serious threat to human health.

Bacteria are found in nature either in free-floating planktonic form or as a communicating community called biofilm.<sup>[1]</sup> Many species are capable of forming biofilms such as *Klebsiella pneumoniae*.<sup>[2]</sup>

Many infections that occur during medical care periods are caused by biofilm development on implantable medical devices.<sup>[1]</sup> The specific structure of biofilm makes bacteria highly resistant to antibiotics and disinfectants.<sup>[3]</sup> Bacteria included in biofilms seem to be insensitive to biocidal agents compared to their planktonic forms. Concentrations required to inhibit biofilms have been reported to be up to 1000 times more than those inhibiting planktonic forms.<sup>[3]</sup>

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Therapeutic failures and the substantial cost of treating infections caused by resistant bacteria require alternatives to conventional therapeutic agents.<sup>[4]</sup>

Plant chemicals are among these substitutes, and many studies have shown interesting antibacterial and antibiofilm activities of plant extracts. Due to their important biodiversity, plants are rich in secondary metabolites.<sup>[4]</sup> In Algeria, a significant number of vegetal species are used for medicinal purposes.<sup>[5]</sup>

This work aims to evaluate the efficiency of polyphenolic extract of *Pulicaria crispa* in preventing biofilm formation by *K. pneumoniae* obtained from respiratory catheters.

## MATERIAL AND METHODS

### Plant

*P. crispa* was collected from the Algerian Sahara in El Hoggar region in Tamanrasset. GPS coordinates are as follows: N22, 298106 and E5, 622164. The plant was identified by botanists at Mouloud Mammeri University in Algeria. It was harvested in January 2018 with more interest to flowery tops, cleaned and washed to remove residues, and dried and crushed in fine powder.

### Polyphenols

Polyphenols were extracted from 30 G of the powder by maceration during 72 H in a mix of methanol-formic acid 95%–5%, respectively (Sigma-Aldrich). The macerate was filtered, evaporated at 35°C (Rotavapor Stuart), and then stored at 4°C.<sup>[6]</sup>

### Dosage of polyphenols

From a standard solution of gallic acid (1 mg/ml), dilutions were realized: 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 mg/ml. Simultaneously, for the polyphenols, dilutions  $\left( SS, \frac{SS}{2}, \frac{SS}{4}, \frac{SS}{8}, \frac{SS}{16}, \frac{SS}{32}, \frac{SS}{64} \right)$  were prepared (SS: stock solution).

A volume of 1.58 ml of distilled water was mixed with 10 µl of calibration range dilution or the sample and 100 µl of the Folin–Ciocalteu reagent (Sigma-Aldrich). At ambient temperature and obscurity, 300 µl of Na<sub>2</sub>CO<sub>3</sub> were added, and the whole was left 2 H. Absorptions were measured at 765 nm (Biotech Engineering Management Co. Ltd.). The concentration of polyphenols is expressed in equivalent mg of gallic acid.<sup>[7]</sup>

### High-performance liquid chromatography analysis

Phenolic compounds were separated on a C18 (150 mm, 4.6 mm, and 5 µm) column at 25°C. The mobile phase contains water, acetic acid, and methanol in a gradient mode with a rate of flow of 1 ml/min (YLClarity–Céhydroatography SW). The obtained chromatograms at 254 nm were compared

with those obtained with standards of some well-known phenolic compounds.

### Bacterial strains

Strains were obtained from bronchial catheters of patients with low respiratory infections. They were identified by their biochemical characters and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-SM). The *K. pneumoniae* ATCC 700603 was used as a positive control.

### Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

From a young culture, a colony was deposited in double on the target plate (Bruker Daltonics TM) and then covered with the prepared matrix solution. The whole was dried and analyzed. The obtained spectra were compared to a database (Maldi Biotyper 3.0. Bruker Daltonics, Germany). A good identification of the species is confirmed if the scores are higher than 1.9.<sup>[8]</sup>

### Preparing bacterial inoculum

Young colonies were emulsified in physiological sterilized water. The obtained suspension had a charge near 10<sup>8</sup> UFC/ml. Another inoculum was prepared using heart–brain broth (CONDA) instead of physiological water to assess the minimal inhibitory concentration of biofilm CMIBs.<sup>[9,10]</sup>

### Antibacterial activity and minimal inhibitory concentrations

Antimicrobial activity was assessed by depositing 10 µl of the crude extract and dilutions (MICs) on a 6-mm disc which was deposited on an inoculated Petri dish. Gentamicin and ciprofloxacin (Liofilchem) were used as a control following the Clinical and Laboratory Standards Institute method.<sup>[9,10]</sup>

### Biofilm formation and antibiofilm activity

Young colonies were added to 10 ml of brain–heart infusion broth (BHIB) with 2% of saccharose and then incubated. Tubes were cleaned using phosphate-buffered saline (PBS) at pH = 7.3 (Metrohm 620) and then dried. Tubes undergo coloration by crystal violet 0.1% during 15 min and then washed and dried in upside-down position. There is a biofilm forming only when a film covers the walls and the bottom of tubes.<sup>[11]</sup>

MICB assessment using the phenolic extract of *P. crispa* was realized in 96-well microplate. We added 100 µl of the inoculum with BHIB supplemented with 2% of saccharose and then incubated the microplate. Floating bacteria were eliminated from wells by aspiration, and wells are then rinsed three times using PBS. After that, 30 µl of every dilution and 70 µl of tryptic soy broth were added according to O'Toole protocol with some modifications.<sup>[12]</sup> One line of

the microplate was used for the positive control and another one for the negative control.

After incubation of the microplate, wells were emptied and rinsed three times with PBS to remove surplus, dried then colored with crystal violet. After that, it was washed again and dried.

Reading was accomplished by ELISA microplate reader at 590 nm (Elisa Plate Analyzer).<sup>[12]</sup> A very low absorption shows that biofilm formation was inhibited by polyphenolic extract.

The mean absorbance was used to express results, and biofilm inhibition was determined using the following equation:

$$\text{Specific biofilm formation} = \frac{\text{OD sample} - \text{OD negatif control}}{\text{OD positif control}}$$

Values of specific biofilm formation (SBF) less than positive control indicate a biofilm inhibition. The lower it is, the more the extract acts to inhibit biofilm formation.<sup>[13]</sup>

### Statistical analysis

Data were represented using arithmetic means and standard deviation of means (Excel 2016 and IBM SPSS Statistics 25.0) and studied using one-way analysis of variance where  $P \leq 0.05$  was taken as statistically significant.

## RESULTS

### Polyphenolic extract

The extraction yield was  $14.51\% \pm 1.94\%$ , and the concentration was  $6.80 \pm 0.81$  mg gallic acid equivalent (GAE) per G of dry matter. Table 1 provides the polyphenol composition obtained by high-performance liquid chromatography (HPLC).

Major components include quercetin, caffeic acid, and isoferulic acid. The rest of components were found in moderate or small amounts: rutin, ferulic acid, luteolin, and protocatechuic acid [Figure 1].

### Bacterial identification

The identification of ten strains by MALDI-TOF-MS reveals values higher than 1.9, confirming that the obtained bacteria belong to *K. pneumoniae* species [Table 2].

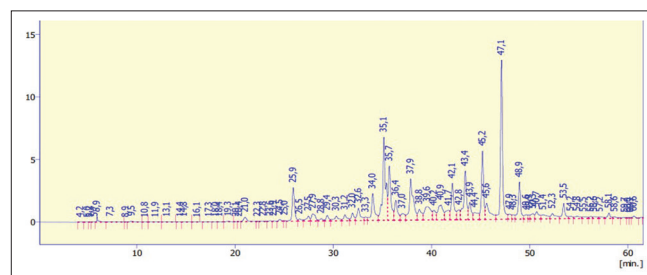
### Antimicrobial activity and Minimal Inhibitory Concentrations

Antimicrobial activity of the polyphenolic extract and antibiotics against *K. pneumoniae* was found important. Inhibition zone values vary between  $12.55 \pm 0.31$  and  $24.00 \pm 0.02$  mm, and MIC values range from 0.1 to 0.425 mg/ml [Table 3].

**Table 1: Composition of polyphenols**

Compound	Rt (min)
Hydroxytyrosol	10.8
Gallic acid	17.3
Salicylic acid	23.5
Proanthocyanidin dimer	23.6
Protocatechuic acid	26.5
Caffeic acid	27.7
Catechin	30.3
p-coumaric acid	34.00
Caffeic acid	35.1
Ferulic acid	37.9
Myricetin-3-o-glucoside	41.7
Rutin	43.4
Isoferulic acid	45.2
Rosmarinic acid	45.6
Quercetin	47.1
Quercetin 3-B-o-galactoside	47.9
Benzoic acid	43.4
Apigenin 7-o-glucoside	58.3
Luteolin	48.9
Ellagic acid	50.7

Rt: Retention time



**Figure 1:** High-performance liquid chromatography analysis of *Pulicaria crispera* polyphenols

### Biofilm formation and antibiofilm activity

From all strains, five are strongly forming biofilms, four are moderately, and one is not forming any biofilms.

Results indicate a high antibiofilm activity for six strains: a moderate one for two strains and a low activity for one strain with CMIBs between 0.21 and 3.4 mg/ml [Table 4].

Figure 2 shows the absorbance results. Figure 3 shows the rate of inhibition. SBF values range from 0.004 to 0.077 for the stock solution. They increase with the dilution of the extract until they reach that of the positive control (0.753), where it represents a negative activity.

## DISCUSSION

Antimicrobial and antibiofilm activities of plant extracts have been highlighted in several studies. Exploiting these encouraging results could lead to resolve the bacterial

**Table 2: Values of the obtained scores by the MALDI-TOF-SM**

Strain	1	2	3	4	5	6	7	8	9	10
Score	2.157	2.323	2.036	2.131	2.18	2.222	2.172	2.125	2.086	2.2

**Table 3: Inhibition zones and minimal inhibitory concentrations**

Strain	Polyphenols		Antibiotics IZ (mm)	
	IZ (mm)	MICs (mg/ml)	Gentamycin	Ciprofloxacin
1	/	/	S (9.23)	R
2	18.55±0.09	0.1	R	R
3	12.08±0.31	0.21	R	R
4	19.64±0.1	0.425	S (16.00)	R
5	23.00±0.05	0.1	S (12.76)	S (11.00)
6	18.81±0.22	0.425	R	R
7	/	/	R	R
8	22.1±0.08	0.21	S (18.9)	S (16.08)
9	19.5±0.15	0.21	S (19.9)	R
10	22.00±0.08	0.1	S (19.00)	S (12.95)
C +	24.00±0.02	0.1	S (23.00)	S (12.00)
C-	/	/	/	0.089±0.0103

Values for the IZ are represented by mean±SD of triplicate analysis. SD: Standard deviation, IZ: Inhibition zone, R: Resistant, S: Sensitive, MIC: Minimal inhibitory concentration, /: Not tested

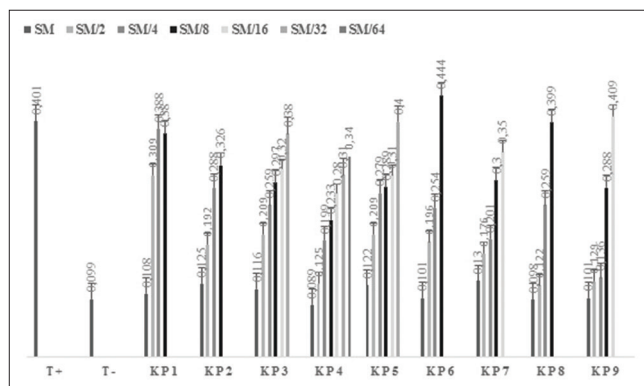
**Table 4: Biofilm formation, MICBs, and optic density**

Strain	Biofilm formation	Antibiofilm activity	MIBCs (mg/ml)	OD 590 nm
1	+++	+	3.40	0.108±0.011
2	+++	+++	0.85	0.288±0.09
3	+++	+++	0.425	0.32±0.109
4	++	+++	0.21	0.31±0.078
5	+++	+++	0.425	0.309±0.1
6	++	++	1.7	0.254±0.004
7	+++	+++	0.21	0.3±0.036
8	++	++	1.7	0.259±0.071
9	++	+++	0.85	0.288±0.099
10	/	/	/	/
C +	+++	/	/	0.401±0.009
C-	-	/	/	0.089±0.0103

Values for the OD at the CMIBs are represented by mean±SD. OD: Optic density, +++: High formation, ++: Moderate formation, -: Negative, SD: Standard deviation, MIBCs: Minimal Inhibitory Concentration of Biofilm, /: Not tested (the strain is not biofilm forming)

resistance and biofilm formation.<sup>[4]</sup> The formation of biofilms on medical devices is a serious public health problem.<sup>[1]</sup>

Few studies on the assessment of the inhibitory action of polyphenols on biofilm formation are available in literature. Moreover, no studies have been carried out on the antimicrobial and antibiofilm activities of *P. crispa* in the Saharan species. This endemic species is used by the local population for antimicrobial and antiseptic properties. Furthermore, the arid zone exposes the plant to stress leading it to synthesize more secondary metabolites responsible of different biological activities.<sup>[14]</sup>

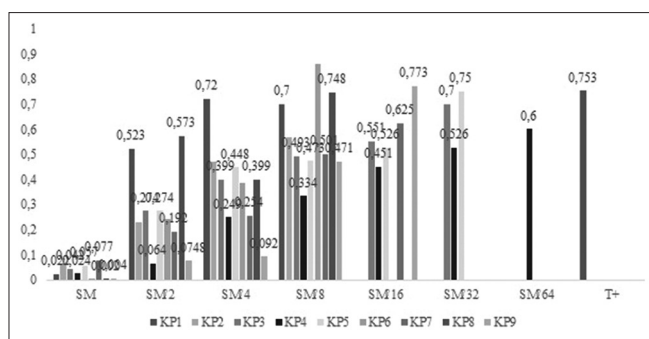


**Figure 2:** Values of absorbances for each well in the microplate reader. Assays were carried out in triplicate, and the results were expressed as mean values ± standard deviation.  $P < 0.05$  was considered statistically significant

The present work focuses on strains of *K. pneumoniae*, currently found in respiratory infections and having a high ability to form biofilms. It should be noticed that 82% of nosocomial infections are caused by biofilm-forming bacterial species on medical devices.<sup>[1]</sup>

Strains of *K. pneumoniae* were identified by MALDI-TOF-MS, reported as the best method to identify bacteria by determination of peptide fingerprints.<sup>[8]</sup> Any natural or artificial surface may be the site of bacterial colonization and formation of biofilms. This is the case for all varieties of biomedical implants.

The transition from a planktonic to biofilm form is regulated by many factors. The authors highlighted that biofilms have mechanisms acting against antimicrobial agents and the defense means of the host.<sup>[3]</sup>



**Figure 3:** Rate of biofilm inhibition

The qualitative tube method was used to test the ability to form biofilms by strains. This method seems to be easy, despite some difficulties in reading results.<sup>[11]</sup>

The results of this study are in accordance with another work using the same bacteria isolated from urinary catheters. All strains were biofilm forming.<sup>[2]</sup> This shows that *K. pneumoniae* is an extremely biofilm-forming bacterium under different conditions.

In addition, methanol was used since it is the best solvent to extract antimicrobial substances. Its polarity allows extracting polar and moderately polar antimicrobial molecules.<sup>[4]</sup>

The inhibition of nine strains of *K. pneumoniae* biofilm formation was demonstrated by the methanolic extract of *P. crispa* with contractions varying from 0.21 mg GAE to 3.40 mg GAE. Inhibition concentrations of planktonic forms vary between 0.1 and 0.425 mg GAE. These results are encouraging because biofilms of the same bacteria were inhibited by gentamicin at 10 mg/ml, while their planktonic form was inhibited at 0.512 mg/ml.<sup>[2]</sup> For the same antibiotics, some strains used in this work are resistant while they are sensitive to the action of the polyphenolic extract at lower concentration (0.1 mg/ml). These activities could be explained mainly by the chemical composition of the methanolic extract.

HPLC showed the presence of proanthocyanidin dimer which reduces to small concentration the slim production and thus inhibits the formation of biofilm.<sup>[15]</sup> Ellagic acid is also present, and it inhibits bacteria and biofilms by destructing the cell membrane.<sup>[16]</sup>

Gallic acid is revealed by HPLC, and it inhibits glucosyltransferase and fructosyltransferase leading to biofilm inhibition.<sup>[17]</sup> Rosmarinic acid acts against planktonic forms and young stages of biofilms.<sup>[18]</sup> Catechins infiltrate cells by destructing the lipidic bilayers.<sup>[19]</sup>

Flavonoids such as apigenin, luteolin, quercetin, and rutin act by inhibiting nucleic acid synthesis<sup>[20]</sup> and energy

metabolism<sup>[21]</sup> or other mechanisms such as inhibiting of autoinducer mediated cell-cell signaling.<sup>[22]</sup>

Furthermore, it should be noticed that some flavonoids such as rutin can inhibit the formation of biofilm without inhibiting the planktonic form,<sup>[23]</sup> such as the case of strains 1 and 7.

## CONCLUSION

This study examines some bacterial strains of *K. pneumoniae* isolated from respiratory infections in patients with bronchial intubation. This bacterium has a high capacity to form biofilms causing spread of nosocomial infections.

Thus, the study aimed to evaluate the capacity of biofilm forming by some strains of *K. pneumoniae* and the efficiency of the polyphenolic extract of *P. crispa* in preventing biofilm formation. These strains showed the ability to form biofilms on intubation probes and resistance to many antibiotics.

Results show that the polyphenolic extract contains quercetin as a major component. It prevents the formation of biofilms in all studied strains and inhibits 80% of planktonic forms.

Finally, although many plant extracts have already shown their antimicrobial and antibiofilm activities, their application in clinical cases may require a long process. Other tests such as toxicity testing, pharmacokinetic and pharmacodynamic studies, drug interactions, and side effects should be accomplished in future researches.

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## Conflicts of interest

There are no conflicts of interest.

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