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Antimicrobial activity of dried fig (*Ficus carica* L.) extracts from the region of Mascara (Western Algeria) on *Enterobacter cloacae* identified by MALDI-TOF/MS

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ABSTRACT: *Enterobacter cloacae* is currently known as a urinary tract infection agent, especially in hospitals recognized by its resistance to 3rd generation cephalosporin's, which makes it a target for different works in order to find natural and definitive means of fight and treatment. Their limited biochemical reactivity and their different morphotypes is a real obstacle to their identification by conventional phenotypic means. 16S rRNA and 18S rRNA gene sequencing is highly successful for bacterial identification. However, in recent years, matrix-assisted laser desorption ionization time in flight mass spectrometry (MALDI-TOF MS) has emerged as a very valid technique for the identification and diagnosis of microorganisms. Our study aims to identify three bacteria belonging to the *Enterobacter cloacae* species isolated from various environments by the MALDI-TOF/MS method and then to study their antimicrobial activity against some extracts of dried figs of *Ficus carica* fruits grown in the mascara region (western Algeria). The determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) shows a significant inhibition of the activity of *E. cloacae* by the methanolic extract of El-Keurt variety at 2.34 mg/ml of extract. This study seems to give good guidance for the use of dried figs against *Enterobacter* infections.

Keywords: *Enterobacter cloacae*; *Ficus carica*; Antimicrobial activity; Dried figs; MIC; MBC.

1. INTRODUCTION

The fig tree (*Ficus carica* L.) is a dicotyledonous tree of the *Moraceae* family [1]. It is one of Algeria's three main fruit productions: Olive, Fig and Citrus. The Algerian fig orchard with nearly 7.6 million trees is still one of the main fruit species in the country and constitutes more than 10% of the national arboreal heritage [2]. According to United State Department of Agriculture (2013), *F. carica* exceptionally the dried form contained bioactive compounds such as arabinose, β-β-amyrins, carotins, glycosids, β-setosterols xanthotoxol. Fig is an important resource of vitamins, minerals, water and fat and the highest plant sources of calcium and fiber [3].

The fruits and leaves of *Ficus carica* are traditionally used to treat diseases as hemorrhoids linked to constipation, play a stimulating role, laxative, cough suppressant, emollient, resolver, emmenagogue [4] and to regulate hyper cholesterolemia [5].

The existing battle continues between humans and the multitude of microorganisms causing infection and disease. Tuberculosis, malaria and more recently the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS epidemic) have affected a significant portion of the human population, causing significant morbidity and mortality. Around the middle of the 20th century, the development of antibacterial drugs demonstrated the efforts and the human resources used to control these infections. With respect to bacterial infections, the situation improved dramatically with the availability of penicillin for use in the early 1940s. Almost as soon as the antibacterial drugs were deployed, the bacteria responded with various forms of resistance. As the use of antimicrobials increased, so did the level and complexity of resistance mechanisms exhibited by pathogenic bacteria [6].

For that purpose, it must be both reliable and fast, according to the clinical conditions. The automation of bacterial identification previously relied on the inclusion of biochemical tests in miniaturized media, then requiring integrated reading and interpretation in the system [7]. Currently microorganisms are best identified using 16S rRNA and 18S rRNA gene sequencing. However, in recent years MALDI-TOF/MS has emerged as a potential tool for microbial identification and diagnosis [8].

Enterobacter cloacae complex (ECC), species found in the environment, is a commensal of the human gastrointestinal tract. Able to shift from a commensal state to that of opportunistic pathogen, it has become of clinical importance because of its increasing implication in infections among intensive care unit patients (prevalence of 5–10%) [9].

Viewing the alternative properties of *F. carica*, our study deals with different extracts of dried figs grown in three different regions of Mascara (El-Keurt, Ain Fares and Sidi Bendjebbar) to investigate their antibacterial activity against three species of *Entrobacter cloacae* isolated from different sources: fecal matter, urine and wastewater (sewage) identified by MALDI-TOF/MS.

2. MATERIALS AND METHODS

2.1. Plant samples

The figs used were cultivated in three region of the Mascara commune (El-Keurt, Ain Farés and Sidi Bendjebbar) all described in Table 1 during December 2014. The varieties were confirmed by the Technical Institute of Mascara Fruit Trees (ITAF). The drying was carried out according to the traditional method under the sun away from dust and rodents.

Table 1. Description of the three varieties of figs and harvest areas.

Region	Color	Latitude	Longitude	Altitude	Climate
El-Keurt	Black	35°22′51.61″	0°5′30″	529 m	semi-arid, dry and cold
Ain Farés	Green	35°28′47.62″	0°14′41.55″	804 m	semi-arid, dry and cold
Sidi Bendjebbar	Yellow	35°25′29.27″	0°8′26.28″	738 m	semi-arid, dry and cold

2.2. Extract preparation

Aqueous and methanolic extracts were prepared from 50 g of plant powder macerated in 200 ml of methanol or 100 ml of distilled water with continuous stirring for 24 hours. The mixture was filtered and concentrated with rotavapor at 40°C under vacuum to obtain a dry extract [10].

2.3. Determination of the antibacterial activity

Antibacterial activity was determined by antimicrobial susceptibility using the diffusion method on agar medium [11]. The three microorganisms were isolated from fecal matter, urine and sewage collected from the laboratories of microbiology of Yessad Khaled and Meslem Tayeb hospitals (Mascara, Algeria) and identified using the MALDI-TOF/MS.

2.4. Paper disk method

Paper disks impregnated with methanolic and aqueous extract already dissolved in 10% DMSO [12] were then deposited on the surface of the Muller Hinton agar previously inoculated with *Enterobacter cloacae* species with suspensions of 10^6 germs/ml. A negative control disc was impregnated with 10 µl of different solvents used in each experiment. Positive control discs of Gentamicine 10 µl/ml were also included. All the plates were incubated at 37°C for 24 hours. Microbial growth was determined by measuring the diameter of the zone of inhibition and the mean values were calculated [13, 14].

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial activity was performed by a serial dilution technique using 96 well microliter plates [15]. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. The minimum bactericidal concentration (MBC) was determined on the basis of the lowest concentration of aqueous or methanolic extract that kill 99.9% of the test bacteria by plating out onto each appropriate agar plate. Bacterial species were cultured from 18 hours cultures and suspensions were adjusted to 0.5 McFarland standard turbidity [16]. A dilution series was performed in the wells ranging from 150 µg/ml to 1.17 µg [17].

2.6. Antimicrobial effects of combined extracts

The antimicrobial combinations assay included *Ficus carica* extracts plus gentamicin. The (FICI) is the sum of the FICs of each of the drugs which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone [18]. The interpretation of the results was based on the growth of bacteria at different concentrations of combined extracts to conclude the synergistic effect, cumulative, indifferent and antagonistic according to the FIC values: FIC $\leq$ 0.5, FIC = 0.5-1, FIC = 1-4 and FIC $\leq$ 4 respectively [19].

3. RESULTS AND DISCUSSION

3.1. Antibiogram method

After a multitude of cultural, macroscopic, microscopic and biochemical tests, 13 isolates belonging to the species *Enterobacter cloacae* were isolated. The three targeted strains were selected according to their antibiotic resistance. The results of the antibiogram are grouped in the Table 2.

According to the table 2, the selected strains indicate a remarkable resistance to the antibiotics used. The diameters of the inhibition zones obtained vary from 7 to 15 mm.

The Antibiotic Committee of the French Society of Microbiology (2018) mentioned that a bacterium is considered to be resistant if the diameter of the zone of inhibition is less than 8mm, intermediate if the diameter is between 8-22 mm. More than 22 mm, the bacterium is considered sensitive to the antibacterial agent. According to these criteria, the three strains appear to have total or intermediate resistance against the antibiotics used.

Table 2. Diameters (in mm) of the inhibition zones obtained by antibiogram method.

	CN 10	NA 30	Aug 30	PI 20	CI 30	RA 5	AX 25	SXT 25	PT 15	E 15	OX 10	P 10	SP 100	NTX 30	AM 10	CTX 25	L 20	DO 30
1	12	9	7	10	9	7	R	R	R	R	R	10	R	13	R	9	10	7
2	R	8	11	15	10	R	12	R	9	7	12	R	10	15	R	10	8	7
3	R	11	R	14	R	R	9	13	R	R	R	11	7	10	7	13	12	R

1: urinary tract infection, 2: food poisoning, 3: wastewater.

CN: Gentamycin (10 mg/ml), NA: Nalidixic Acid (30 mg/ml), Aug: Augmentin (30 mg/ml), PI: Pipemidic Acid (20 mg/ml), CI: Ciprofloxacin (30 mg/ml), RA: Rifamycin (5 mg/ml), AX: Amoxicillin (25 mg/ml), STX: Sulfamethoxazole + Trimethorpine (25 mg/ml), PT: Pristinamycin (15 mg/ml), E: Erythromycin (15 mg/ml), OX: Oxillin (10 mg/ml), OX: Oxillin Penicillin G (10 mg/ml), SP: Spiramycin (100 mg/ml), NTX: Nitroxoline (30 mg/ml), AM: Ampicillin (10 mg/ml), CTX: Cefotaxime (25 mg/ml), L: Lincomycin (20 mg/ml), DO: Doxycyclin (30 mg/ml), R: resistant (no visible zone).

3.2. MALDI-TOF/SM identification

The MALDI Biotyper software measures the very abundant proteins present in all microorganisms. The characteristic motifs of these proteins are used to accurately and reliably identify a particular microorganism, by comparing the tested model against a large open database, to determine the identity of the microorganism down to the level of the species [20].

The identification of the strains by MALDI-TOF/SM indicates that the three selected strains really belong to the *Enterobacter cloacae* species with technique scores varying from 2.375, 2.019, and 2.128 respectively (Figure 1).

According to the manufacturer's criteria, A score between 2.33 and 3 is considered as a very probable species identification, between 2 and 2.32 as a good genus identification and a probable species identification [21], between 1.7 and 1.999 as a likely gender identification, requiring further testing. Scores below 1.699 do not allow identification and the sample must therefore be retested or subjected to other tests [22].

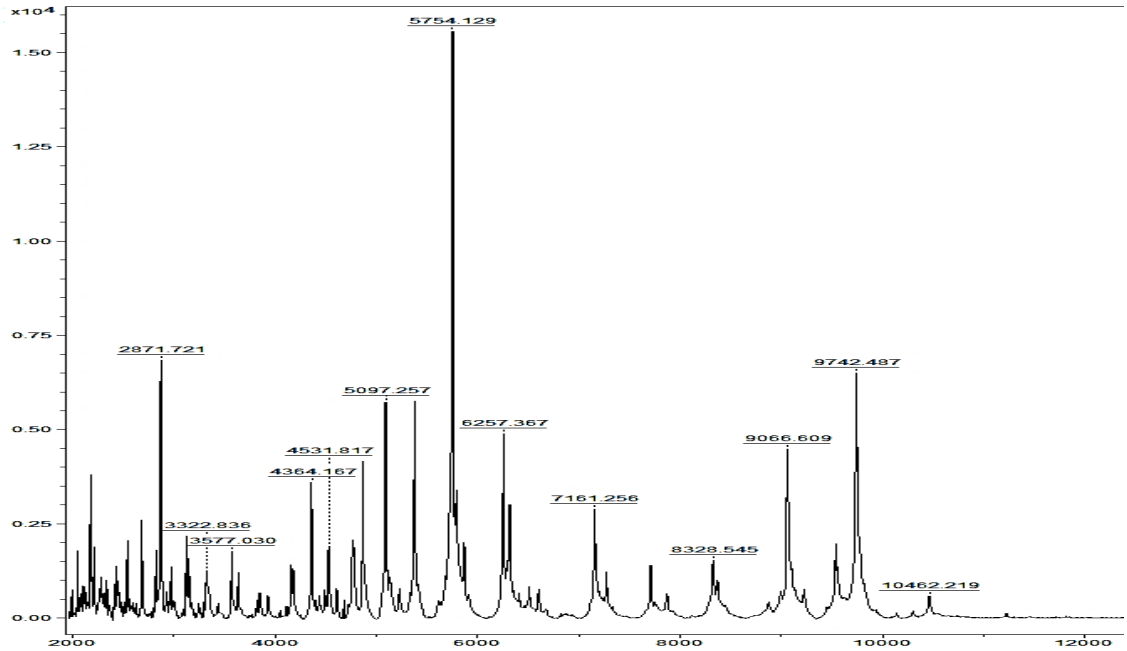

Figure 1. Spectrogram of the strain *Enterobacter cloacae* (1) isolated from the urine of a patient with urinary tract infection.

The identification of Enterobacteria is generally very reliable by the MALDI-TOF/MS seen the very high score rate recorded [23, 24]. The study of Gram-negative bacilli seems fairly straightforward, because their thin wall provides good protein extraction performance, and their spectra are therefore quite rich [25].

3.3. Antibacterial activity

Antibacterial activity was investigated by an antimicrobial susceptibility test using the paper disk method by measuring zone of inhibition. Our results indicated an important antimicrobial activity of methanolic extracts especially that of the Sidi Bendjebbar variety. The dried fig of *F. carica* extract was found to inhibit *Enterobacter cloacae* species (Table 3).

Table 3. Diameters (in mm) of the inhibition zones obtained by paper disk method.

Strain	Ampicillin (40 µg/ml)	El-Keurt		Sidi Bendjebbar		Ain Fares	
		Aqueous	Methanolic	Aqueous	Methanolic	Aqueous	Methanolic
1	15	12	15	15	17	10	16
2	19	12	17	15	16	12	16
3	16	10	16	13	17	11	17

1: urinary tract infection, 2: food poisoning, 3: wastewater.

All *Ficus carica* extracts exhibited an antibacterial activity against *Enterobacter* species at different levels. The inhibition values on these bacteria were in the range of 10 to 17 mm, while methanolic extracts were the most active against these tested bacteria.

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC).

Strain	Ampicillin (40 µg/ml)		El-Keurt				Sidi Bendjebbar				Ain Fares			
			Aq		Meth		Aq		Meth		Aq		Meth	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	2.34	75	4.68	150	2.34	75	4.68	300	4.68	150	37.75	600	9.37	300
2	1.17	75	2.34	300	2.34	75	18.75	600	37.75	300	37.75	600	18.75	150
3	2.34	150	9.37	300	2.34	300	37.75	150	1.17	75	9.37	300	4.68	150

All concentrations are in mg/ml.

The second strain of *Enterobacter cloacae* was the most sensitive germ with a MIC range from 1.17 mg/ml to 37.75 mg/ml. The aqueous extracts was the less effective especially Ain Fares variety. The results of the antibacterial activity showed that the MeOH extract of *F. carica* fruits exhibited strong activities against *Enterobacter cloacae*.

4. DISCUSSION

The development of new antimicrobial agents and antibiotics seems essential because microorganisms developed resistance to many drugs leading to a considerable increase in the death rate from infectious diseases [26]. Plants have long been used for the treatment of infectious diseases such as asthma, sexually transmitted infections, skin infections and many others [27].

The objective of this study was to evaluate the antibacterial activity of six extracts of *Ficus carica* fruits, on growth of *Enterobacter cloacae* isolated from three different sources. This strain was always among the strains revealing a remarkable resistance to the various plant extracts testified by others [28, 29].

To overcome the problems related to classical phenotypic species identification methods, this study evaluated the capability of MALDI-TOF/MS to identify these species [30, 31]. Biochemical identification by API gallery was chosen as a method of reference. MALDI-TOF/MS identified *Enterobacter* strain with a high log (score) of 2.375, showing the possible ability of this method to differentiate the species within this complex. Because of the high sensitivity of this technical in detecting peptides and the possibility of identifying bacteria with software such as Biotyper, it has been possible to demonstrate the high quality of identification [8].

Analysis of these results indicates that the three microbial strains tested are all sensitive to different extracts. For each extract, this sensitivity results in a decrease in number of colonies of germs tested with increasing concentration. Tests carried out on E. cloacae showed that this bacterium is sensitive to plant extracts regardless of its origin isolation. Methanolic extracts hold the most potent antibacterial activity against the tested microorganisms than the aqueous extracts exceptionally those of El-Keurt variety with an MIC equal to 2.34 mg/ml. These results agree with several works [26, 32].

Several recent studies report the antimicrobial properties that hold plant extracts to the presence of bioactive compounds and polyphenols [33, 34]. The mechanisms of action of these compounds and their effects on the cell membrane and the wall are known. Their influence on cell permeability in terms interference on functions membranes (electron transport, nucleic acid synthesis and secretion enzymes) would be at the origin of this antimicrobial character [35]. The different antimicrobial activities observed on the three strains tested, although that exercised in a certain relativity are attributable to the presence in these extracts of polyphenolic compounds which act alone or by effect of conjugation or synergy of compounds majority (hydrogenated monoterpenes and oxygenates) and minority compounds (hydrogenated and oxygenated sesquiterpenes) [36].

The FIC method exposed the combination between our *Ficus carica* extracts and gentamycin against the strains studied, the results indicate a interaction between these extracts and the ATB since the FIC index is varied between 0.5 to 1. Many works confirmed this additivity [37].

5. CONCLUSION

Ficus carica fruits have unlimited medicinal potential for the therapy of infection caused by *Enterobacter cloacae* species. Further investigation is necessary to identify those bioactive compounds, which will be a platform for clinical applications.

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